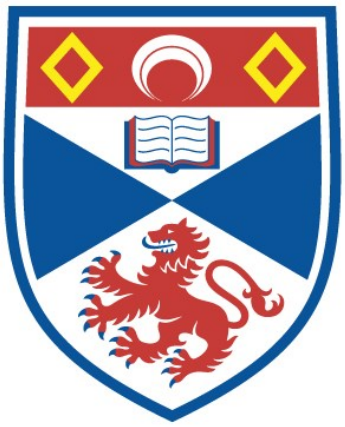
PEA AND RICE SEEDLING SURVIVAL UNDER ANOXIA

Catherine J. Mawer (Mrs S. C. Maberly)

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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PEA AND RICE SEEDLING SURVIVAL UNDER ANOXIA

by

Catherine J. Mawer

(Mrs. S.C. Maberly)

A thesis submitted to the

University of St. Andrews for

the degree of Doctor of Philosophy

Department of Botany

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UNIVERSITY OF ST. ANDREWS

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ABSTRACT

It is not clear whether flood-tolerant plants survive in water-logged soils because they maintain aerobic activity in all regions of the roots, or whether biochemical adaptations to anoxia are involved. Many plants have some capacity for internal ventilation, and so in order to exclude oxygen from the root environment it is necessary to subject both the roots and the shoots to anoxia. Pea and rice seedlings were exposed to anoxia for 1 to 24 h and their tolerances and survival compared.

With pea and rice, cv. Oeiras, tolerance of anoxia decreased as the temperature increased, although rice was more tolerant than pea at a given temperature. Increasing amounts of K^+ were lost from the roots of whole seedlings during anoxia, and seedling recovery on return to air was associated with the seedlings' ability to reabsorb most of the leaked K^+. Aerobic respiration in the root tips was associated with both K^+ uptake by pea seedlings, and the recovery and subsequent growth of pea and three varieties of rice after anoxia. All rice varieties were more tolerant of anoxia at 25°C than was pea at 20°C, but differences between the tolerance limits of the rice varieties were apparent. Although some pea and rice seedlings appeared undamaged after anoxia, the subsequent growth rates of pea and rice, cv. IR8, were seriously impaired.

Ethanol accumulated around the roots of all seedlings during anoxia, but there was no correlation between the quantity of ethanol produced and the different tolerance limits of the seedlings. Pea seedlings that were prevented from transpiring during anoxia were damaged earlier than controls, possibly because of an increase in the rate of accumulation of a toxic product of anaerobic metabolism in these seedlings. Two percent glucose merely delayed, and did not prevent, the onset of damage in whole pea seedlings during anoxia, nor did it enhance the

subsequent growth of the seedlings. Under these conditions, the accumulation of endogenous ethanol to a toxic concentration was considered to have been the more likely cause of seedling death.

From the results presented in this thesis and elsewhere, it is concluded that the immediate cause of seedling death during anoxia is a shortage of substrate for glycolysis, and that the additional effect of ethanol accumulation in some species may reduce their subsequent rate of recovery.

DECLARATION

I declare that this thesis is a record of my own work and that it has not been previously presented in application for a higher degree.

Catherine J. Mawer,

Kendal, April 1982.

CERTIFICATE

I certify that Catherine J. Mawer has spent 12 terms of research under my direction, that she has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967 No.1, and that she is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosphy.

R.M.M. Crawford,

St. Andrews, April 1982.

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CONTENTS

CHAPTER 1

INTRODUCTION

In a well-drained soil, plants obtain much of the oxygen needed for root respiration from the soil air spaces which are interconnected with the atmosphere and so allow oxygen to diffuse into the soil (Gambrell and Patrick,1978). When a soil is flooded, water displaces air from the pore spaces and this greatly reduces both the oxygen content of the soil and the rate of oxygen diffusion into the soil from the atmosphere because the diffusion coefficient of oxygen in water is considerably smaller than it is in air (see Armstrong,1978). Respiration by plant roots and microorganisms can render a submerged soil practically devoid of dissolved oxygen within a few hours (Ponnamperuma,1972), and in a permanently waterlogged soil, as a consequence of anaerobic bacterial activities, a reduced state develops in which the accumulation of potentially harmful substances may occur (Ponnamperuma,1972). However, despite the seemingly inhospitable nature of waterlogged soils, many plant species survive and grow under such conditions. Most agricultural crops, with the exception of rice, are grown on land that is not permanently waterlogged because for most of these species seasonal or transient flooding may result in substantial crop damage or total loss (Kramer and Jackson,1954; Bergman,1959).

Within the plant root, the availability of oxygen determines the fate of the glycolytic end-product, pyruvate, which in the presence of oxygen is oxidised to carbon dioxide and water via the tricarboxylic acid (TCA) cycle, and in the absence of oxygen is either reduced to lactate or decarboxylated to acetaldehyde which in turn is reduced to ethanol (Beevers,1961). The accumulation of lactate or ethanol in plant

roots in an anaerobic environment is considered harmful to the plant b a number of workers (e.g. Fulton and Erickson,1964; Andrews,1977; Prad and Bomsel,1978; Chirkova,1978) presumably because the sustained production of lactate could lead to a lowering of the pH of the cytopl to a dangerous level (Davies, Grego and Kenworthy,1974) and the accumu lation of ethanol may lead to a disruption of membrane permeability (Kiyosawa,1975). In addition to the possible toxic effects of lactate or ethanol during the period of anoxia, there is also the possibility of an energy shortage, because of the inhibition of oxidative phosphor lation, and this may seriously disrupt a number of energy-requiring processes in the cell; aerobic respiration produces 36 moles of adenos triphosphate (ATP) from 1 mole of glucose, whereas in the absence of oxygen only 2 moles of ATP are available from the breakdown of 1 mole of glucose (Beevers,1961).

The main effect of flooding the soil is the removal of oxygen needed for aerobic respiration in the roots, and therefore a sudden reduction in root aeration could have a damaging effect on the growth of species not adapted to waterlogged conditions (Lemon and Wiegand,19 For example, peas suffer extensive injury within a few days of the soi being waterlogged (Cannell et al.,1979; Jackson,1979) yet rice is normally grown in paddy fields where 5-10 cm of standing water is mai tained during the 4-5 months the crop is in the field (Ponnamperuma, 1972). The reasons why some plants can withstand the waterlogging of their roots whereas others suffer injury has been the subject of much research but nevertheless, it is still not clear whether flood-tolerant plants survive because they are able to maintain aerobic activity in all regions of the roots, or whether biochemical adaptations to anoxia are involved.

One of the features of many wetland plants, including rice, i

the possession of large intercellular spaces which form a continuous channel for the diffusion of oxygen from the aerial parts of the plant to the roots (Conway,1937; Barber, Ebert and Evans,1962; Soldatenkov and Chirkova,1963; Armstrong,1967;1975;1978). This internal ventilation provides oxygen for both the maintenance of aerobic metabolism in the roots and the oxidation of phytotoxins at the root surface (Armstrong, 1978). However, although internal ventilation is a property normally associated with wetland species, there are reports that some nonwetland plants are able to transport oxygen from the shoots to the roots (Evans and Ebert,1960; Barber et al.,1962; Heide, Boer-Bolt and Raalte,1963; Greenwood,1967a and b; Greenwood and Goodman,1971; Healy and Armstrong, 1972). Greenwood's data (1967a) showed that the oxygen diffusing from the aerial parts to the roots of several vegetable species (seedlings) was sufficient for root respiration and elongation in an oxygen-free medium, although this may not be the case for established plants of the same species. However, in the case of the flood-tolerant plant root it still remains unclear whether or not the supply of oxygen from the shoots is sufficient to maintain aerobic respiration in the flooded roots. Van Raalte (1940, cited by Barber et al.,1962) showed that the respiratory requirements of rice roots could be fully met by the movement of oxygen down the root but John, Limpinuntana and Greenway (1974), also working with rice, found that the oxygen movement from the shoots to the roots was "insufficient to sustain the full potential of at least some metabolic processes". Furthermore, Mendelssohn et al. (1981) found that root oxygen deficiencies do occur in the wetland macrophyte Spartina alterniflora, although it had previously been shown (Teal and Kanwisher,1966) that internal ventilation was sufficient for aerobic respiration in the roots of this species.

Metabolic differences between the roots of flood-tolerant

and flood-intolerant plants have been found when these plants have been grown for one month with their roots in flooded sand (Crawford,1966; 1967), an increased rate of glycolysis and ethanol production being characteristic of flood-intolerant species. These results led to the suggestion (Crawford,1967) that such flood-intolerant species were excluded from wet areas because of the accumulation of toxic quantities of ethanol in their roots. Flood-tolerant species, on the other hand, did not exhibit an acceleration of glycolysis and accompanying increased production of ethanol, and since then a number of flood-tolerant species have been shown to accumulate non-toxic end-products of glycolysis, such as malate (Crawford and Tyler,1969; McManmon and Crawford,1971), shikimate (Crawford and Tyler,1969) or glycerol (Crawford,1972) instead of ethanol. However, some flood-tolerant species produce ethanol as the end-product of glycolysis e.g. rice (Taylor,1942; Phillips,1947; John et al.,1974) and willow (Chirkova,1978) but in these species ethanol is efficiently removed from the root tissues during flooding. In willow the ethanol that is formed during root anaerobiosis is lost through the lenticels (Chirkova and Gutman,1972) and Bertani, Brambilla and Menegus (1980) reported that 98% of the ethanol produced in rice seedlings subjected to anoxia was found in the growth medium. Ethanol removal in the transpiration stream, as found for sugar beets (Kenefick,1962) and tomatoes (Bolton and Erickson,1970), may also occur. In addition, any oxygen transported from the shoots to the roots would help maintain low root ethanol levels by providing more sites for aerobic rather than anaerobic respiration.

However, the actual cause of damage to flood-intolerant plants when the roots are in flooded soil still remains unclear. Jackson (1979) found that chlorosis and desiccation of the leaves of pea plants produced by waterlogging the roots in a richly organic compost could not be

produced in anaerobic solution culture and unidentified soil toxins may have been involved. Drew and Sisworo (1979) however, reported that early symptoms of flooding damage to 13 day-old barley plants occurred before potential soil toxins had accumulated to harmful concentrations and they concluded that damage to root metabolism may have been caused by the low concentrations of oxygen in the soil. A similar conclusion was reached by Trought and Drew (1980a and b) for waterlogging damage to wheat. Although flood-tolerant species appear to prevent ethanol accumulating in their flooded roots whereas flood-intolerant species do not, ethanol has not been identified as the cause of injury to susceptible plants when their roots are flooded and furthermore, the toxicity of ethanol is itself in doubt (Jackson, pers. comm.).

In both pea and rice seedlings the end-product of anaerobic metabolism is ethanol (Beevers, 1961) yet pea is intolerant of flooding whereas rice is tolerant. In rice, oxygen transported from the shoots to the roots is thought to play a major role in the avoidance of anoxia, and hence of ethanol accumulation, in the flooded roots, although removal of ethanol by exudation and transpiration may also occur. However, if the seedlings are exposed to a completely anaerobic environment the capacity for internal ventilation will be removed. If rice normally relies heavily on the transport of oxygen from the shoots to the roots for its survival in flooded soils, and if ethanol is toxic, then pea and rice seedlings should both be intolerant of anoxia, unless rice possesses a biochemical adaptation for the prevention of ethanol accumulation in its tissues.

This thesis is a record of some responses of pea and rice seedling to conditions of total anoxia. Seedlings were exposed to the anaerobic environment in deoxygenated solutions so that the possible effects of soil toxins were avoided. The toxicity of exogenous ethanol is also

examined. In addition, a brief comparison of the responses to a completely anaerobic environment of two rice varieties, known to have different tolerances to submergence in the field, is also presented.

CHAPTER 2

MATERIALS AND METHODS

2.1 NUTRIENT SOLUTION

A modified Hoagland's solution (Epstein, 1972) was used throughout. One fifth strength solutions were used in work with pea and full strength solutions in work with rice.

2.2 PREPARATION OF PLANT MATERIAL

2.2.1 Pea - Pisum sativum L. cv. Meteor

Dried seeds were soaked for 18 to 20 h in tap water at 20°C in the dark. The imbibed seeds were rinsed twice with tap water and 12 seeds placed to germinate at 20°C in the dark, in a Petri dish on a sheet of Whatman No. 3 filter paper moistened with distilled water. The seeds were watered as necessary with distilled water and any decaying seeds were removed. Usually some seeds had begun to germinate the day after they were placed in Petri dishes - this was called day 0. The majority of seeds had germinated by the next day, day 0-1 (followed by days 1-2, 2-3 etc.). When the roots were 2-3 cm long, and before lateral roots had begun to form, the seedlings were placed in small plastic tubes, containing 5 ml of nutrient solution, by passing the root through a hole in the plastic cap. This method enabled seedlings to be transferred in the caps to different tubes without damaging the developing root systems. Once in the tubes the seedlings were left in the dark at 20°C for 24 h before transference to a growth cabinet at 20°C with continuous illumination at a photon flux area density (PFAD) of 125 μmol m^{-2} s^{-1} (all PFAD measurements refer to the spectral region 400 to 700 nm). In all experiments, unless otherwise stated, pea seedlings were used when 7-8 days old.

2.2.2 Rice - Oryza sativa L. cvs. Oeiras, FR13A and IR8

Oeiras seeds were planted in seed trays containing moist sand, and allowed to germinate in an incubator at 25°C in the dark. The trays were watered daily with tap water and covered with a polythene sheet to reduce evaporation. When the coleoptiles were 2-3 cm long the trays were transferred to a growth cabinet at 20°C, with continuous illumination at a PFAD of 125 μmol m^{-2} s^{-1}. The seedlings were watered daily, usually with tap water but received at least two waterings with nutrient solution before they were used in experiments. In the initial experiments the seedlings were used when 14 days old, day 0 being the day of planting in sand. Later this method was modified because of poor growth at 20°C and seedlings were placed in a growth cabinet at 25°C. In this growth cabinet the PFAD was 220 μmol m^{-2} s^{-1}. In these later experiments, unless otherwise stated, seedlings were used when 10 days old.

Seeds of rice cvs. FR13A and IR8 were obtained from the International Rice Research Institute, Manila, Philippines in December 1980. Before an experiment they were washed thoroughly in tap water to remove the seed dressing. Seeds were germinated and grown at 25°C, as described above for Oeiras, but FR13A and IR8 seedlings were used when 14 days old, day 0 being the day of planting in sand.

2.2.3 Effect of anoxia on seedling growth

In these experiments, 10 day old Oeiras seedlings, 14 day old FR13A and IR8 seedlings and 7-8 day old pea seedlings were used. Pea seedlings were removed from the nutrient solution and weighed before the roots were placed in new tubes containing 5 ml of an appropriate deoxygenated solution ready for anaerobic exposures ranging from 0 to 24 h. Rice seedlings were dug up and their roots washed free of sand in tap water before the seedlings were weighed and treated as described for pea. After the anaerobic exposures, seedlings were removed from the

tubes and planted out in moist sand in seed trays. The trays were placed in a growth cabinet (20°C for pea and 25°C for rice) with continuous illumination at PFADs of 125 and 220 μmol m^{-2} s^{-1} for pea and rice respectively. The seedlings were watered daily, the initial watering with nutrient solution followed by tap water on subsequent days (when seedlings were subjected to anoxia in 2% glucose solutions, the subsequent daily waterings were with tap water containing 2% glucose).

After one week in sand the seedlings were dug up and their roots washed free of sand with tap water. Seedlings were weighed intact and then the roots were excised and weighed separately. The changes in fresh weight were determined and in some cases the mean relative growth rate, $\bar{R}$, was calculated using the following formula:

$$1\text{-}2 \quad \bar{R} = \frac{\log_e W_2 - \log_e W_1}{T_2 - T_1}$$

where: W_1 - initial weight (g)

W_2 - final weight (g)

$T_2 - T_1$ - one week

2.3 ANAEROBIC CONDITIONS

Plants were subjected to an anaerobic environment in an anaerobic incubator (model 1024 /1028 - Forma Scientific Systems, Ohio, U.S.A. - see Plate 2.1) with a gaseous atmosphere comprising 85% nitrogen, 5% carbon dioxide and 10% hydrogen. Any traces of oxygen were removed from the system by reacting with the hydrogen on the surface of a palladium catalyst, forming water which was then absorbed by a desiccant. The plants were kept in the dark during the anaerobic treatments to prevent them from photosynthesising and producing oxygen. Anaerobic conditions

were checked using disposable methylene blue indicators, which remained white in the absence of oxygen and turned blue in its presence.

Deoxygenated distilled water was used in the tubes for all the investigations, including the aerobic controls, and any other test solutions and their aerobic controls were prepared with the deoxygenated distilled water. Initially distilled water was deoxygenated by bubbling with oxygen-free nitrogen, but in later experiments the preparation was carried out in the anaerobic incubator using an aquarium pump to bubble the distilled water with the oxygen-free atmosphere. The deoxygenated distilled water was always freshly prepared by bubbling for 1 or 2 h (depending on the volume) before an experiment.

Most experiments were carried out in the anaerobic incubator at room temperature, which varied from 20 to 24°C. However, in hot weather temperatures as high as 28°C were reached. For precise constant temperature work, plants were kept in anaerobe jars (GasPak Anaerobic Systems, Becton-Dickinson and Co., Cockeysville, U.S.A.) in the dark, in incubators at the desired temperature. These jars were always filled in the anaerobic incubator so that the composition of the anaerobic atmosphere in each experiment was the same. A thin layer of vaseline smeared round the lid of each jar ensured an air-tight seal, and anaerobic conditions were checked using disposable methylene blue indicators.

2.4 MEASUREMENT OF POTASSIUM (K^+)

Immediately before an experiment the roots of 7-8 day old pea seedlings were rinsed thoroughly in two separate washings of distilled water. Fourteen day old rice seedlings (cv. Oeiras) were dug up and their roots washed free of sand in tap water before they were rinsed thoroughly in two separate washings of distilled water. Seedlings of both species were then placed in new tubes containing 5 ml of an

appropriate deoxygenated solution, depending on the experiment, and exposed to aerobic or anaerobic conditions for 1 to 24 h. After these exposures seedlings were either:

(i) removed from the solution immediately, or

(ii) transferred to a growth cabinet at 20°C with continuous illumination at a PFAD of 125 μmol m^{-2} s^{-1} for 24 h (recovery period) before being removed from the solutions.

In both cases the roots were excised, dried at 100°C for 24 h and weighed. The solutions were stored at 4°C in the dark, for K^+ determinations using an atomic absorption spectrophotometer (model A3400, Shandon Southern Instruments Ltd., Camberley, Surrey.).. A standard curve for K^+ was prepared for each analysis, and since most atomic absorption calibration curves become increasingly non-linear at high absorbance values (0.5 to 1.0, A3400 Operator's Handbook), sample solutions were diluted so that their absorbancies fell within the linear portion of the curve.

2.5 MEASUREMENT OF OXYGEN UPTAKE

2.5.1 With the inverted oxygen electrode

The inverted oxygen electrode (Rank Brothers, Bottisham, Cambridge.) is a modification of the Clark-type electrode and was used in conjunction with a Spectro-Plus (M.S.E. Scientific Instruments, Crawley, Sussex.) for measurement of dissolved oxygen. The sample chamber had a built-in water jacket connected to an external constant temperature water bath and was mounted on a base containing the electrode and a magnetic stirrer. A teflon membrane separated the sample chamber and the electrode.

Three ml of 0.1 mol l^{-1} potassium phosphate buffer, pH 6.0 and containing 2% sucrose, was pipetted into the sample chamber and air-saturated at the desired temperature by activating the stirrer (approx-

imately 500 rpm) while the lid was off. When a steady trace was obtained on the chart recorder, the meter was set to read 100% saturation. The stopper was inserted and all the air bubbles excluded from the system through the 1 mm capillary tube drilled up the centre of the stopper. The electrode was then checked for drift by leaving it to run for 20 min in this closed state. If the trace deviated markedly from the straight line a new membrane was prepared and the above procedure repeated until a drift-free trace was obtained. Each new membrane was also tested regularly to check the electrode "leakage" by setting up the meter to read 100% with air-saturated water and then adding a few crystals of sodium dithionite to chemically remove all the oxygen in the solution. The "zero" reading obtained was used to correct the chart scale, although in the majority of experiments electrode leakages were negligible.

The apical 1 cm from three or four root tips, including seminal roots (rice), was excised with a razor blade and placed in the sample chamber on top of a small piece of plastic netting which prevented the roots from fouling the stirrer. The stopper was inserted and all air bubbles excluded. Oxygen uptake by the respiring root tips was followed for 2 h in the dark. The root tips were dried at 100°C for 24 h and weighed, and the rate of oxygen uptake after 1 h was calculated. In later experiments the excised root tips were surface sterilised for 5 min in 0.02% mercuric chloride and then washed twice before being placed in the sample chamber.

2.5.2 With the portable digital oxygen meter

The portable digital oxygen meter and sensor (models 7130 and 7131 respectively, E.I.L. Analytical Instruments, Chertsey, Surrey.) were used to measure dissolved oxygen. The sample chamber was a 500 ml glass reagent bottle immersed in a constant temperature water bath, placed on top of a magnetic stirrer. A stirrer attachment on the end of

the oxygen sensor ensured a constant flow of solution over the membrane, and two built-in thermistors and a platinum-100 temperature probe provided automatic temperature compensation and measurement of the solution.

The sample chamber was filled with 0.1 mol l^{-1} potassium phosphate buffer, pH 6.0 and containing 2% sucrose, which was air-saturated at the desired temperature by aerating for 5 min with an aquarium pump. Whole root systems were attached to small plastic caps containing plasticine, dropped into the bottle and positioned away from the sensor. The caps acted as weights and prevented the roots from fouling the stirrer. A displacement funnel was inserted in the neck of the bottle and the sensor was passed through this and placed in position, displacing excess fluid. Oxygen uptake by the respiring root systems was followed for 2 h in the dark. At the end of the experiment, the excess water in the displacement funnel was carefully pipetted out before the sensor and funnel were removed. The volume of solution in the bottle was measured and the roots were dried at 100°C for 24 h. The rate of oxygen uptake after 1 h was calculated and expressed on a dry weight basis. In later experiments the excised root systems were surface sterilised in 0.02% mercuric chloride and washed twice with distilled water before being placed in the bottle.

2.6 ETHANOL

2.6.1 Enzymatic determination of ethanol

After aerobic or anaerobic treatments, root ethanol content and / or the ethanol content of the solution around the roots were determined enzymatically using a food test-combination kit (Cat. No. 176290, The Boehringer Corporation Ltd., London.) according to the maker's instructions. This determination was based on the oxidation of ethanol in the presence

of alcohol dehydrogenase (ADH) by nicotinamide adenine dinucleotide (NAD), to acetaldehyde with the formation of reduced nicotinamide adenine dinucleotide (NADH), and then the oxidation of the acetaldehyde to acetic acid with the formation of more NADH. The amount of NADH formed in both these reactions was stoichiometric with half the amount of ethanol formed. The quantity of NADH produced was determined by means of its absorption at 365 nm using a spectrum-line photometer equipped with a mercury vapour lamp (Eppendorf-Gerätebau, Hamburg, Germany.), and so the amount of ethanol present in the extract could be calculated.

2.6.2 Preparation of extracts

Seedlings were prepared and subjected to aerobic or anaerobic conditions as described in Sections 2.2 and 2.3. Root ethanol content and /or the ethanol content of the solution around the roots were determined as follows:

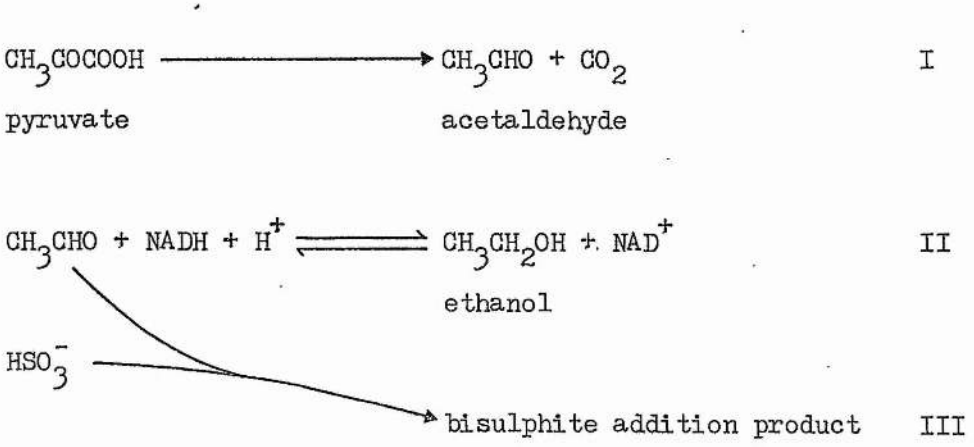
Root ethanol content - immediately after removal from the aerobic or anaerobic environment the seedling was removed from the tube and the root excised and weighed as quickly as possible before being held in liquid nitrogen for 20 sec. The frozen root was then placed in 4 ml of ice-cold 6% perchloric acid and stored in a deep freeze at -20°C until required. On removal from the deep freeze the samples were partially thawed before being ground up in 4 ml of ice-cold 6% perchloric acid in a chilled pestle and mortar. The resulting suspension, the total volume of which did not exceed 25 ml including rinsings, was centrifuged for 30 min at 4°C at 1200 rpm (1800 g). The supernatant was poured into a 50 ml beaker, surrounded with ice, and neutralised with 5.0 mol l^{-1} potassium carbonate solution added drop-wise. One drop of methyl orange solution was added as indicator and at the colour change from pink to yellow, the total volume of the resulting suspension was measured. This suspension was left in a refrigerator at 4°C for 1 h, after which time

the supernatant was decanted and assayed for ethanol.

Ethanol content of the solution - after removal from the aerobic or anaerobic environment the seedling was removed from the tube and the undiluted solution assayed for ethanol immediately. The root was excised, dried at 100°C for 24 h and weighed. However, if the root ethanol content was to be determined as well, then the tube of solution was stoppered and left in ice for not more than 5 min before it was assayed while the root was prepared for storage as described above.

2.6.3 Inhibition of ethanol production

In higher plants under anaerobic conditions, pyruvate is decarboxylated to acetaldehyde (I) which is then reduced to ethanol (II). However, in the presence of sodium bisulphite ($NaHSO_3$), the bisulphite moiety reacts with acetaldehyde to form a bisulphite addition product (III, Metzler,1977).



The removal of acetaldehyde not only inhibits ethanol production by removing the substrate for reaction II , but also inhibits the regeneration of NAD^+, a compound essential in the earlier stages of glycolysis, contributing further to the inhibition of ethanol production.

Pea seedlings were subjected to aerobic and anaerobic conditions in solutions of $NaHSO_3$ ranging from 10^{-5} to 10^{-2} mol l^{-1}. After these

treatments the solutions were assayed for K^+ and ethanol as described in Sections 2.4 and 2.6 respectively. In some cases root ethanol content was also determined (Section 2.6.2).

2.6.4 Preparation of ethanol solutions

A 1.0 mol l^{-1} ethanol solution was prepared from a stock solution containing from 0.7904 to 0.7935 g ethanol per ml at 20°C, and all subsequent dilutions were made from this molar solution. All dilutions were made with distilled water. The stock solution was replaced regularly in 75 ml quantities and kept in a small stoppered conical flask in a desiccator to minimise deterioration of the solution, caused mainly by water absorption. For anaerobic investigations solutions were prepared with deoxygenated distilled water, but for growth experiments ordinary distilled water was used.

2.6.5 Effect of ethanol on seedling growth

In these experiments pea seedlings were used when 3-4 or 4-5 days old, before lateral roots had begun to form. These seedlings and 10 day old rice seedlings (cv. Oeiras), all of which had had their roots washed free of sand with tap water, were weighed and then placed in plastic tubes containing 5 ml of an ethanol solution - distilled water served as the control. Ethanol treatments were for 1, 3 or 5 days in a growth cabinet with continuous illumination at PFADs of 125 µmol m^{-2} s^{-1} for pea at 20°C, and 220 µmol m^{-2} s^{-1} for rice at 25°C. All the solutions were changed every 12 h and the ethanol solutions were freshly prepared from the stock solution on each occasion. After the ethanol treatments the seedlings were removed from the tubes and weighed again. The seedlings were then planted out in moist sand in seed trays and replaced in the appropriate growth cabinet. The seedlings were watered daily, the initial watering with nutrient solution followed by tap water on subsequent days. After one week in sand the seedlings were dug up

and their roots washed free of sand with tap water before the seedlings were weighed. The changes in fresh weight were determined and the mean relative growth rate, $\bar{R}$, was calculated using the formula given in Section 2.2.3.

2.7 MISCELLANEOUS (PEA)

2.7.1 Prevention of transpiration

In these experiments pea seedlings were germinated and grown as described in Section 2.2.1. Immediately before the seedlings were exposed to the aerobic or anaerobic environment (see Section 2.3), either the upper and lower surfaces of the leaves were sprayed with an anti-transpirant (S600 - "The Christmas Tree Spray", Synchemicals Ltd., London), or the shoot system was excised with a razor blade immediately below the lowest leaf. After the aerobic or anaerobic exposures, the K^+ content of the solutions were measured as described in Section 2.4. In the case of intact seedlings sprayed with S600, the appearance of the leaves was noted but for seedlings without shoot systems (detopped seedlings) this was impossible.

2.7.1 Dark-grown seedlings and /or cotyledon removal

Seedlings were germinated and grown as described in Section 2.2.1. However, the dark-grown seedlings remained in the dark at 20°C until they were 7-8 days old, and after their anaerobic treatments these seedlings were placed in the dark for their 24 h recovery period.

With both dark-grown and light-grown seedlings the cotyledons were excised with a razor blade immediately before the aerobic or anaerobic exposures.

Plate 2.1 Anaerobic Incubator

(Forma Scientific Systems, Ohio, U.S.A.)

CHAPTER 3

TOLERANCE OF ANOXIA

3.1 INTRODUCTION

In the field, rice plants are normally grown under flooded conditions with the roots subjected to an anaerobic environment and the shoots in air (Ponnamperuma, 1972), while peas suffer extensive injury within a few days of the soil being waterlogged (Cannell et al., 1979; Jackson, 1979). Experiments to investigate the early symptoms of flooding injury to susceptible plants, by placing the root systems of whole plants in pots of flooded soil or in anaerobic solutions, have shown that in some flood-intolerant species the exclusion of oxygen from the root environment is sufficient to induce symptoms of flooding injury (Letey, Stolzy and Blank, 1962; Willey, 1970; Drew and Sisworo, 1979; Trought and Drew, 1980 a and b).

The ability of many flood-tolerant species to survive in waterlogged soils has been attributed to their efficient internal ventilating systems which allow aerobic conditions in the roots of these plants to be maintained (Armstrong, 1964; 1978). Taylor (1942) concluded that in the complete absence of oxygen "rice seedlings were able to make more nearly normal amounts of growth than wheat seedlings" probably as a result of "differences in the anaerobic activities of the two types of seedlings". Similarly, Vartapetian, Andreeva and Nuritdinov (1978) attributed the ability of six day old rice coleoptiles to grow in an anaerobic environment to an active metabolic adaptation, although they attributed the survival of rice plants in flooded soils to the ability of the plants to aerate their roots rather than to metabolic adaptations to anoxia in the root cells. Young pea and rice seedlings were therefore

subjected to anoxia and their responses compared.

It is known that a loss of substances from root cells is enhanced when the roots are subjected to certain adverse conditions, such as chilling temperatures (Christiansen, Carns and Slyter,1970) or an anaerobic environment (Vlamis and Davis,1944; Grineva,1962; Marschner, Handley and Overstreet,1966; Hiatt and Lowe,1967; Christiansen et al.,1970). Chilling injury can be measured by the amount of ion efflux from thawed tissues, injury being proportional to the conductivity of the water (Levitt,1972; Patterson, Murata and Graham,1976) and the extent of electrolyte leakage from some imbibing leguminous seeds is correlated with subsequent seed death in the field (Matthews and Bradnock,1968).

Potassium is the major cation in plant cell fluid and since it was the principle cation lost from chilled tissues (Patterson et al.,1976) it was assumed that it would probably also account for a large proportion of any electrolyte leakage occurring from roots under anaerobic conditions. Measurement of K^+ has also been used to assess the effect of organic acids on the extent of ion leakage from roots (Lee,1977).

Pea and rice seedlings were subjected to conditions of total anoxia in deoxygenated solutions, and the K^+ content of the solution was measured either immediately after the anaerobic exposure, or after a 24 h recovery period in air. Growth experiments were carried out to discover whether or not the K^+ results were correlated with the ability of the seedlings to recover.

3.2 RESULTS

3.2.1 Tolerance limits under anoxia at 20°C

Seven to eight day old pea seedlings and 14 day old rice seedlings cv. Oeiras were subjected to anoxia for 1 to 24 h inclusive (Section 2.3) and after a 24 h recovery period in air the seedlings were photographed.

Plate 3.1 shows that after anaerobic exposures of up to and including 10 h, pea seedlings appeared to have been unaffected by the absence of oxygen but after 11 h anoxia some disruption of normal cell functioning had taken place. Anaerobic exposures of 10 h or less resulted in pea seedlings with green, turgid leaves, and the solutions around the roots of these plants remained clear. However, anaerobic exposures of 11 h or more resulted in seedlings with dark green, flaccid leaves, and the solutions around the roots of these plants were cloudy. The time where the sudden change in seedling appearance occurred is referred to as the cut-off. In contrast to pea seedlings, rice seedlings appeared to have been unaffected by similar anaerobic treatments (see Plate 3.2). Here the shoots remained green and turgid and the solutions around the roots were clear after all the anaerobic exposures, presumably because rice is better adapted to survive under conditions of low or zero oxygen.

3.2.2 Diagnosis of injury

The K^+ content of the solution around the roots was measured either immediately after the anaerobic treatment or after a 24 h recovery period in air (Section 2.4). Figure 3.1 shows that increasing amounts of K^+ were lost from the roots when whole pea and rice seedlings were subjected to increasing lengths of time under anoxia. Potassium leakage from pea roots was positively correlated with the length of the anaerobic exposure between 4 and 18 h anoxia, but after 18 h anoxia the leakage values remained steady. Potassium leakage from rice roots was also correlated with the length of time under anoxia, but only between 0 and 17 h and after this time the leakage values remained steady. It is interesting to note that these two ecologically distinct species exhibited a similar response when placed under conditions of total anoxia. Whole pea and rice seedlings remaining in air for similar lengths of time did not exhibit an increasing leakage of K^+. However, in some cases K^+ was

detected in the solutions but the quantities obtained were usually less than 30 μmol $(gdw)^{-1}$, and the K^+ probably diffused out of the free space.

Figure 3.2 shows the net K^+ in solution after the seedlings had experienced a 24 h recovery period in air. The solutions around the roots of pea seedlings that had been exposed to anoxia for up to and including 8 h contained less than 50 μmol K^+ $(gdw)^{-1}$, whereas solutions from around the roots of seedlings that had been exposed to anoxia for 9 h or longer contained at least 180 μmol K^+ $(gdw)^{-1}$, the quantity of K^+ increasing as the length of the anaerobic exposure increased. The time at which the sudden increase in the K^+ content of the solution occurred, i.e. between 8-9 h anoxia in this experiment (see Figure 3.2), is also referred to as the cut-off and it coincided with the observed symptoms of damage. The solutions around the roots of rice seedlings contained less than 40 μmol K^+ $(gdw)^{-1}$ after anaerobic exposures of up to and including 21 h, but between 22 and 24 h anoxia more than 60 μmol K^+ $(gdw)^{-1}$ were detected in the solutions and this increase, although small, may represent the cut-off for rice. However, visual symptoms of damage, such as wilting, were not apparent in any of the rice seedlings but as rice grew poorly at 20°C - the leaves were often beginning to turn yellow at the tips before the seedlings were exposed to anoxia - it is possible that any further damage to these plants caused by the anaerobic treatments was masked by their already unhealthy appearance. Temperatures of 25°C and above are normally used in experiments with rice and therefore further investigations were carried out at this temperature, when growth of rice seedlings was much improved.

Pea seedlings were also subjected to anoxia in calcium sulphate ($CaSO_4$) solutions at a concentration of 0.5 mmol l^{-1}, to determine if calcium ions altered membrane permeability and hence K^+ leakage. These results are in Figure 3.3. The visual cut-offs in this experiment were

between 2-3 h anoxia for both the water control and the $CaSO_4$ treatment. However, the analysis of net K^+ in solution after a 24 h recovery period in air revealed two possible cut-offs, i.e. between 5-6 and 7-8 h anoxia, neither of which coincided with the observed symptoms of damage. During this particular experiment the temperature in the anaerobic incubator was extremely unstable and ranged from 20 to 25°C. The duration and time of occurrence of the higher temperatures are unknown and it is possible that these temperature fluctuations were responsible for the differences between the visual cut-off and that determined by K^+ analysis. Nevertheless, it is clear from the results that the presence of calcium ions did not delay the onset of damage.

3.2.3 Growth and recovery after anoxia

With pea seedlings the visual cut-off and the cut-off indicated by measurement of the net K^+ remaining in solution after a 24 h recovery period, with the exception of the calcium experiment, were always the same i.e. only seedlings reabsorbing most or all of the leaked K^+ appeared to have been undamaged by their anaerobic treatments. The cut-off occurred within the space of one hour and the difference between damaged and undamaged seedlings was distinct - there was no gradual appearance of the symptoms. Similarly treated seedlings were therefore planted out in sand and their growth after one week measured (Section 2.2.3). These results are in Figure 3.4. In this experiment the visual cut-off occurred between 12-13 h anoxia, but although the 1 to 12 h treatments inclusive appeared undamaged, when the seedlings were planted out in sand it became apparent that even short periods of anoxia had seriously impaired their growth. After the cut-off slight increases in fresh weight were observed in 14, 15 and 16 h treatments as a result of renewed shoot growth of these seedlings, although their roots were brown and flaccid. Plate 3.3 shows the appearance of similarly treated seedlings after one

week in sand.

The effect of anoxia on the fresh weight changes of rice seedlings was investigated at 25°C (Section 2.2.3) and these results are expressed as the mean relative growth rate, $\bar{R}$, in Figure 3.5 with the previous results for pea seedlings included for comparison. A possible cut-off for rice occurred between 14-16 h anoxia, since after 14 h anoxia there was a sudden decline in $\bar{R}$ and associated with this was a change in the appearance of the seedlings. Up to and including 14 h anoxia rice seedlings were of similar appearance with upright, green shoot systems, whereas after 16 h anoxia seedlings had yellow shoot systems, some of which were wilted and dried.

At 20°C rice seedlings appeared to have been unaffected by anoxia until after 21 h (Figure 3.2) but at 25°C they appeared to have been damaged after 14 h anoxia (Figure 3.5). The effect of temperature on the tolerance of pea seedlings to anoxia was therefore investigated to discover if pea seedlings were similarly affected.

3.2.4 Relative tolerances of pea and rice seedlings to anoxia

Figure 3.6 shows the effect of temperature on K^+ leakage from the roots of whole pea seedlings subjected to anoxia. As the temperature increased, more K^+ was lost from the roots and the cut-off occurred earlier; at 5 and 15°C all the seedlings recovered from all the anaerobic exposures, at 20°C the cut-off occurred between 6-8 (either 6-7 or 7-8) h anoxia, but at 25°C none of the seedlings recovered. Again the visual cut-off and the cut-off determined by measurement of net K^+ after a 24 h recovery period in air, occurred at the same time. Thus, damage to pea seedlings as a result of anoxia occurred earlier as the temperature of the anaerobic exposure was increased. Similarly, increasing the temperature from 20 to 25°C appears to have affected rice seedlings in the same way, since observations for rice at 25 and 20°C were similar to

those for pea at 20 and 15°C respectively.

Temperature fluctuations in the anaerobic incubator probably caused the variations in the times of cut-off and in the quantities of K^+ lost by pea seedlings in replica experiments that were performed on different occasions.

3.3 DISCUSSION

Differences between the tolerance limits of pea and rice seedlings cv. Oeiras subjected to anoxia at 20°C were apparent, but the initial responses of the flood-intolerant and the flood-tolerant species were the same. Both species lost K^+ from the roots during anoxia, in agreement with the findings of other workers (Vlamis and Davis, 1944; Marschner et al., 1966; Hiatt and Lowe, 1967) but contrary to some reports (Hiatt and Lowe, 1967; Christiansen et al., 1970) the presence of calcium ions did not reduce the loss of K^+ during anoxia. However, the fluctuating temperature may have influenced this result. The increase in membrane permeability during anoxia has been attributed to the reduced rate of energy supply, which is thought to be insufficient to maintain membrane integrity (Simon, 1974). Pea roots lost more K^+ than rice roots either because pea seedlings contained more K^+ than rice seedlings, or because their membranes became more permeable during the anaerobic treatment. Vlamis and Davis (1944) reported that below 3% oxygen, K^+ loss from the roots of barley and tomato plants was great, whereas loss from rice roots was only slight, but no explanation was given.

Potassium losses from pea roots increased with increasing temperature, and observations of both pea and rice seedlings indicated that tolerance of anoxia was reduced as the temperature was increased. Kramer and Jackson (1954) found that increasing the soil temperature from 20 to 34°C accelerated the rate and extent of injury to flooded tobacco plants

and Varade, Stolzy and Letey (1970) found that a low oxygen supply was more detrimental to shoot and root growth of wheat at 32.6°C than at either 25.1 or 18.3°C, presumably because at higher temperatures the respiration rate was increased and a low oxygen supply could not satisfy the oxygen demand. In these present experiments in the absence of oxygen, the increasing temperatures may have caused increases in the metabolic rate which resulted in toxic quantities of ethanol accumulating more rapidly, hence the earlier cut-offs at higher temperatures.

After a 24 h recovery period in air at 20°C, only pea seedlings experiencing anoxia for 8 h or less recovered from their anaerobic treatments, whereas rice seedlings experiencing up to and including 21 h anoxia appeared to have been undamaged by the anaerobic exposures at this temperature (Figure 3.2). However, if the tolerances of pea and rice seedlings to anoxia are compared at temperatures more suitable for the growth of the individual species, i.e. 20°C for pea and 25°C for rice, rather than at the same temperature, it becomes apparent that rice is not much more tolerant of conditions of total anoxia than is pea. The results in Figure 3.5 show that the abilities of the two species to recover after their anaerobic treatments were markedly different in that pea seedlings showed a decline in $\bar{R}$ during one week in sand following 2-12 h anoxia, whereas rice seedlings maintained a steady $\bar{R}$ during the same period following similar anaerobic treatments. If ethanol is toxic to plant tissues, then these differences may reflect species differences in the rate of production and /or accumulation of ethanol. In the case of pea, the decline in $\bar{R}$ may result from the accumulation of ethanol in the tissues in increasingly damaging quantities. In rice, the rate of ethanol production during anoxia may just exceed its rate of removal from the tissues so that a damaging concentration is not reached until after 14 h anoxia. The continued growth of rice

seedlings after 16 h anoxia may result from the continued growth of the coleoptile, frequently cited as the only plant organ able to grow during anoxia (Opik,1973; Pradet and Bomsel,1978; Vartapetian et al., 1978). Recently however, Echinochloa crus-galli, a problem weed of rice fields, has also been shown to germinate and grow under anaerobic conditions (Kennedy et al.,1980). Taylor (1942) found that rice coleoptiles were more tolerant of exogenous ethanol than rice roots and it is possible that a similar situation exists with regard to their tolerance of endogenous ethanol.

A flood-tolerant plant relying solely on oxygen transport from the shoots for the avoidance of anoxia in flooded roots might be expected to sustain injury similar to a flood-intolerant plant when both plants are subjected to a completely anaerobic environment. With pea and rice seedlings cv. Oeiras this was not the case and rice seedlings were found to be more tolerant of anoxia than pea seedlings, presumably because of differences in their metabolism during anoxia.

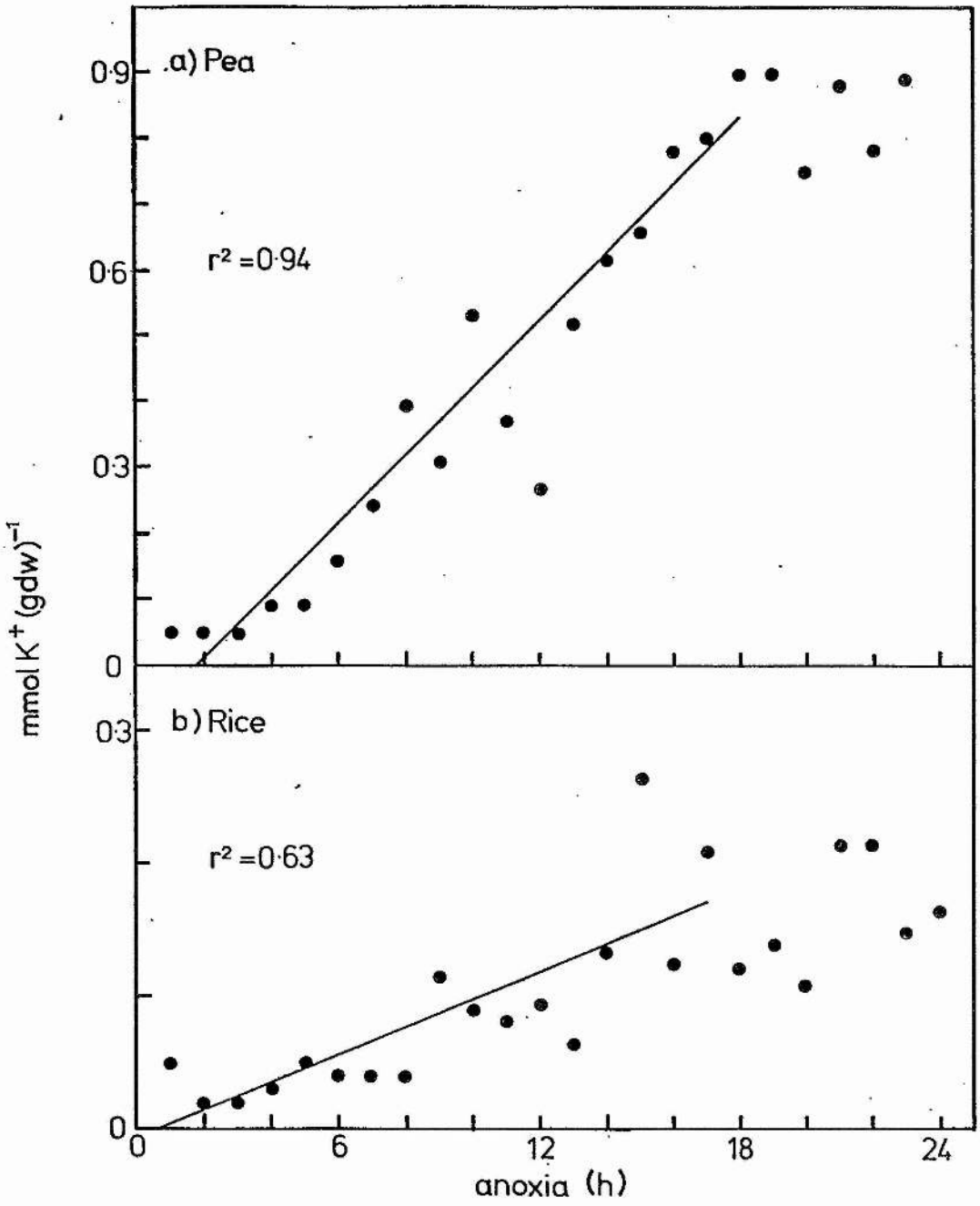


Figure 3.1 The effect of different lengths of time under anoxia on K^+ in the solution around the roots of intact seedlings (20°C).

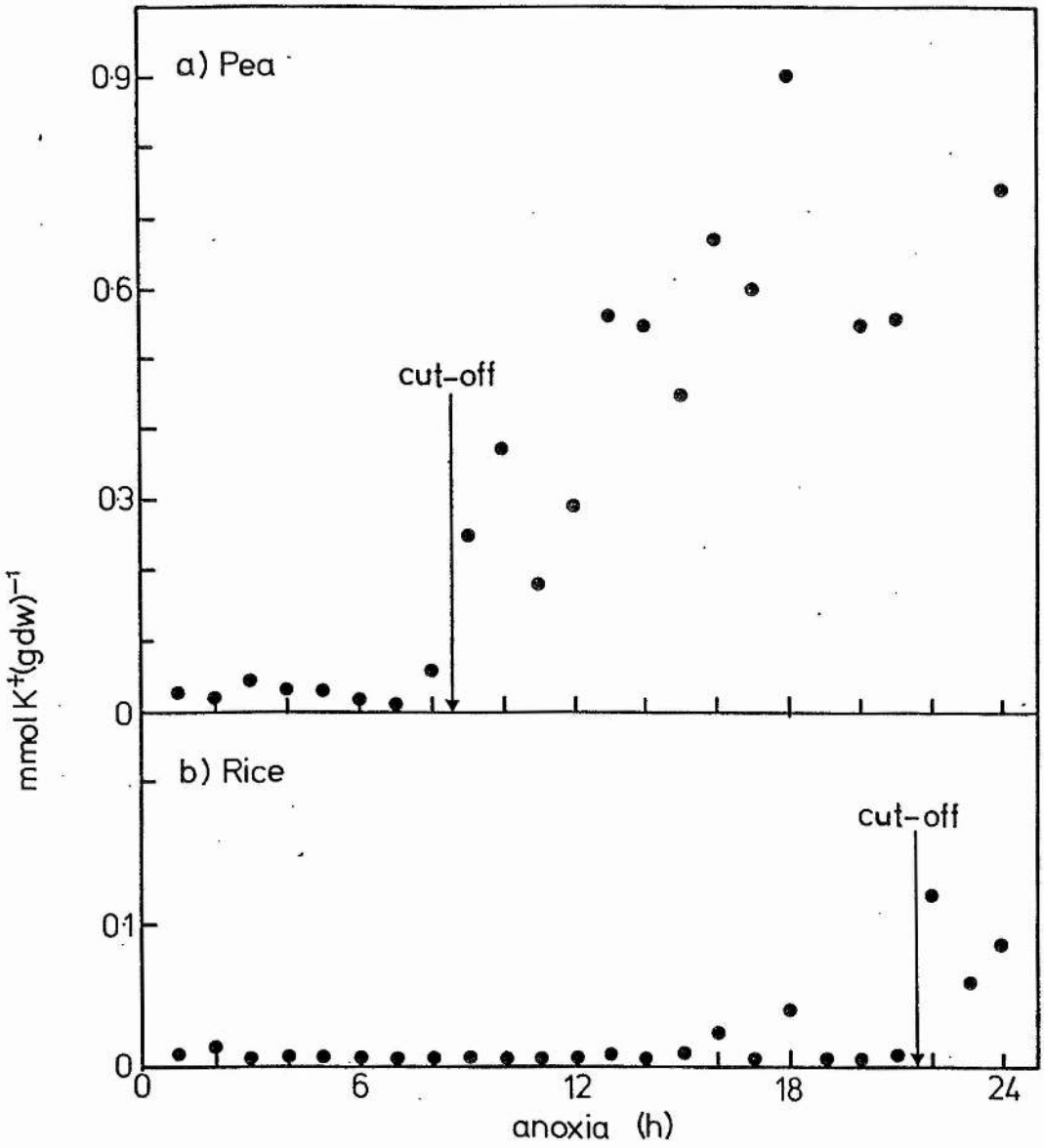


Figure 3.2 The effect of different lengths of time under anoxia followed by a 24 h recovery period in air, on the K^+ remaining in the solution around the roots of intact seedlings (20°C).

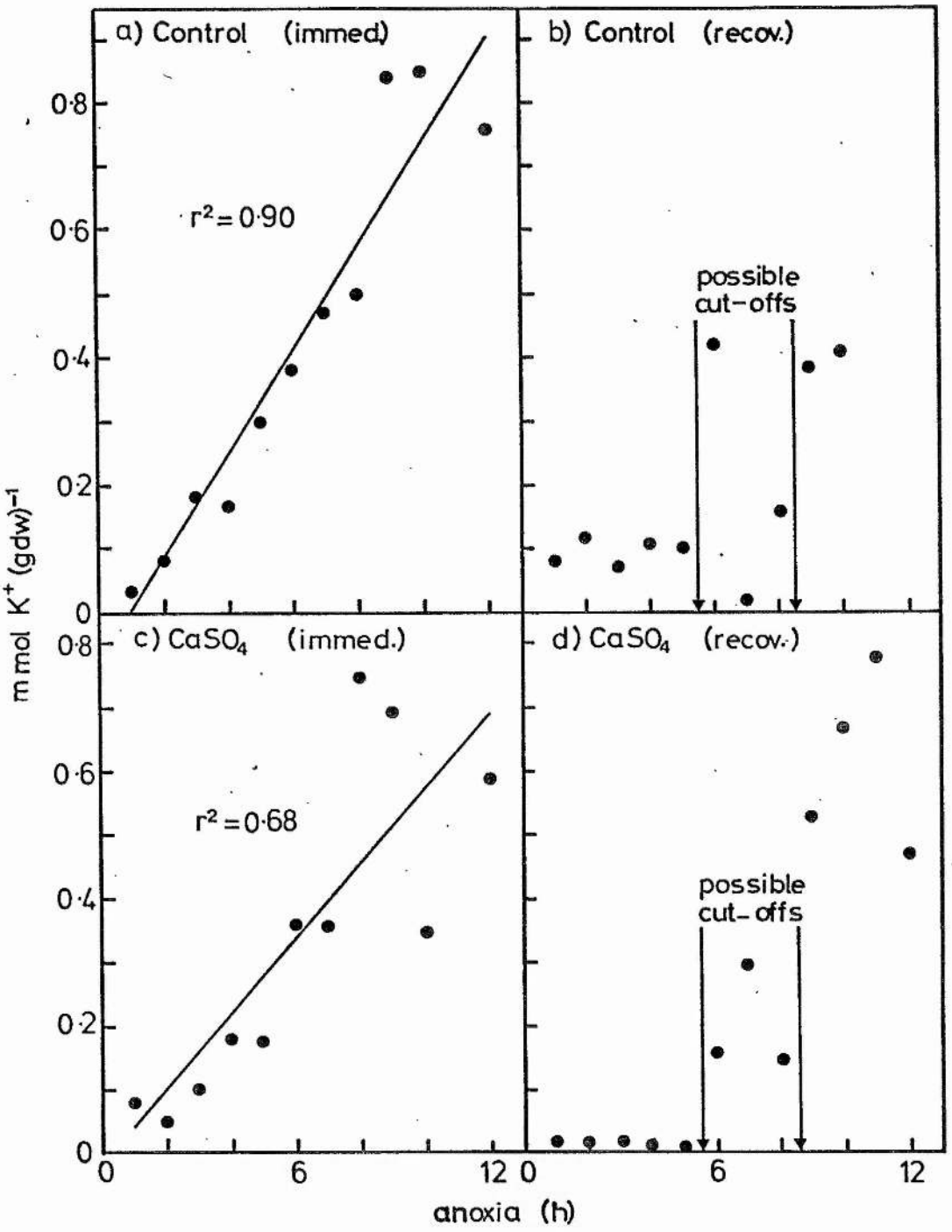


Figure 3.3 The effect of $CaSO_4$ (0.5 mol l^{-1}) on the K^+ in the solution around the roots of intact pea seedlings after different lengths of time under anoxia (a and c) and after a 24 h recovery period in air (b and d).

Figure 3.4 The effect of different lengths of time under anoxia on the subsequent changes in fresh weight of pea seedlings after one week in sand.

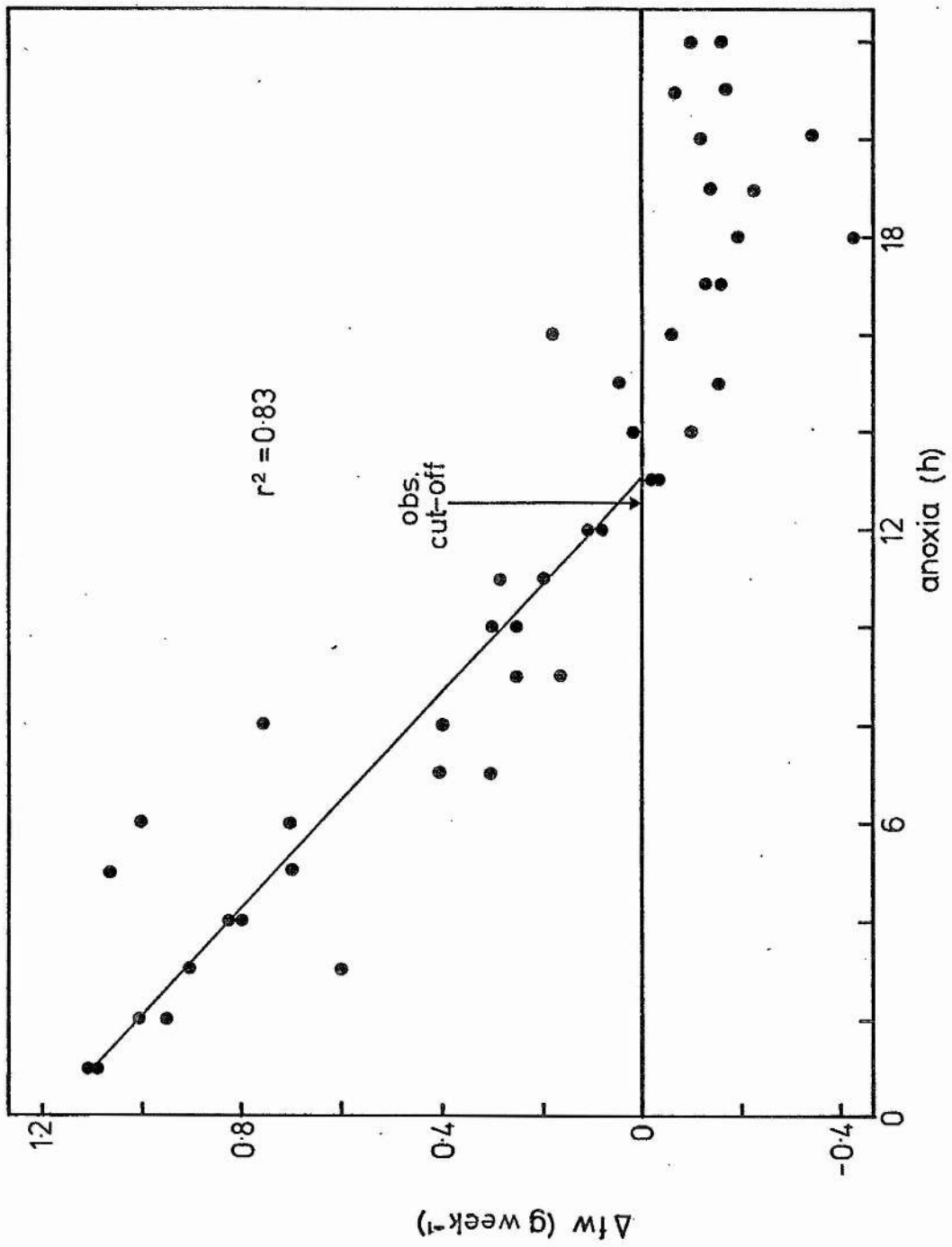
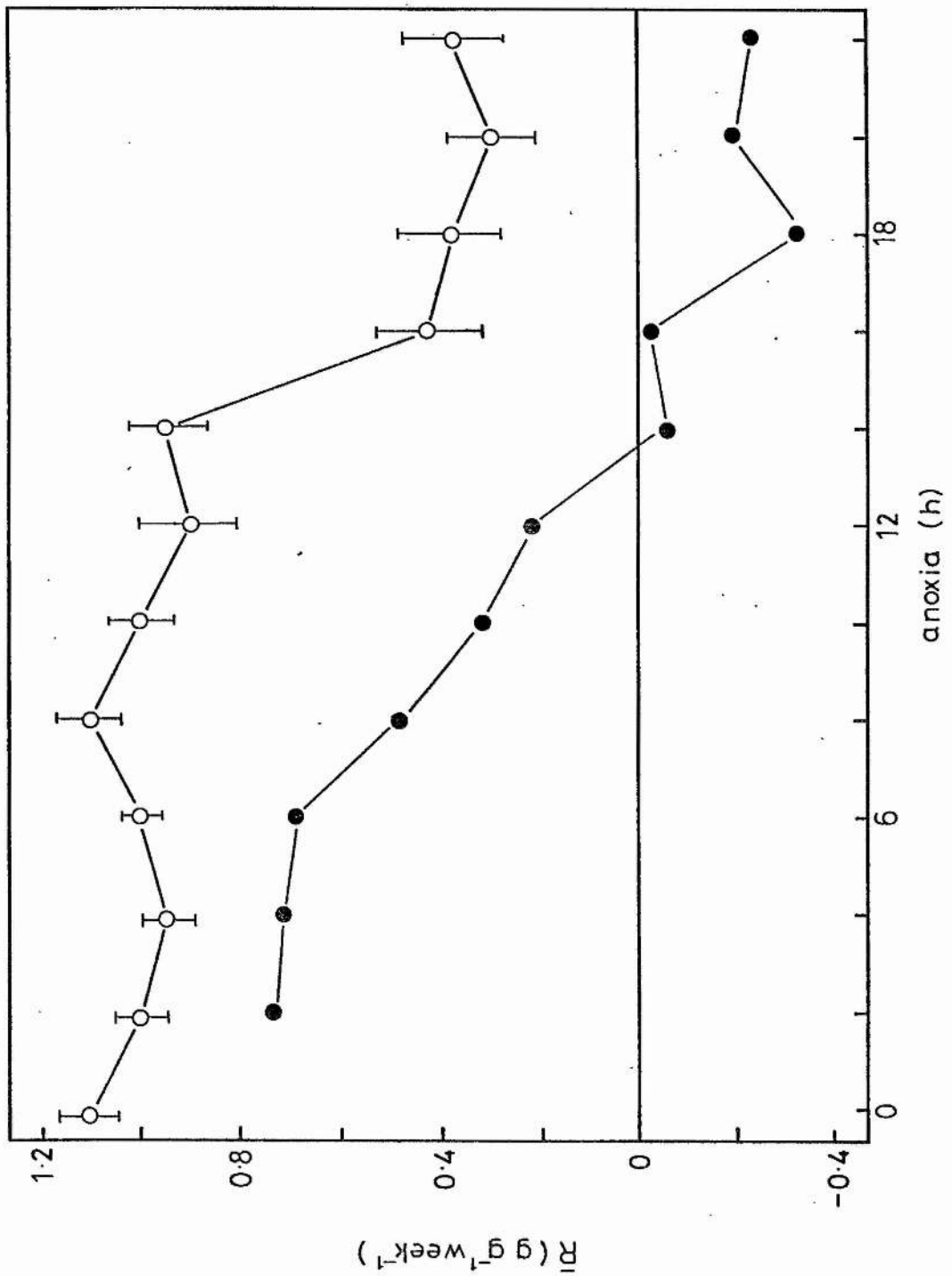

Δ fw (g week⁻¹)
1·2
0·8
0·4
0
-0·4
0
6
12
18
anoxia (h)
r² = 0·83
obs.
cut-off

Figure 3.5 The effect of different lengths of time under anoxia on the subsequent mean relative growth rates, $\bar{R}$, of seedlings after one week in sand.
Pea (●) 20°C, mean of 2; Rice (O) 25°C, mean of 6 ± standard error.


1·2
0·8
0·4
0
-0·4
0
6
12
18
anoxia (h)
$\overline{R}$ (g g^{-1}week^{-1})

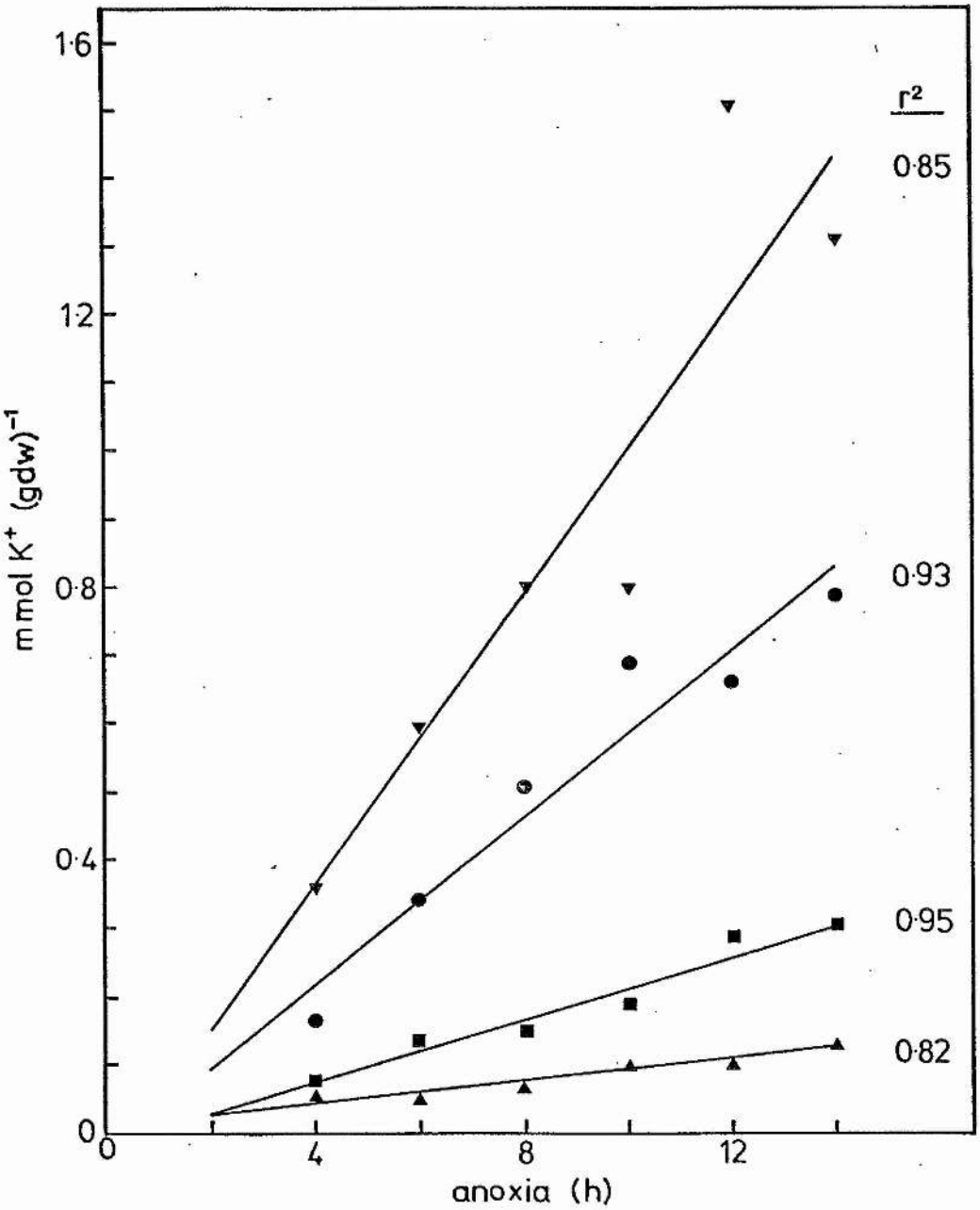


Figure 3.6 The effect of temperature on K^+ loss from the roots of intact pea seedlings during anoxia.

▲ 5°C, ■ 15°C - all seedlings recovered;

● 20°C - only the 4 and 6 h treatments recovered;

▼ 25°C - none of the seedlings recovered.

Plate 3.1 The effect of different lengths of time (h) under anoxia followed by a 24 h recovery period in air, on the appearance of pea seedlings (20 - 24°C). The visual cut-off is between 10 and 11 h anoxia.

Plate 3.2 The effect of different lengths of time (h) under anoxia followed by a 24 h recovery period in air, on the appearance of rice seedlings (20 - 24°C). The seedlings were unaffected by these anaerobic exposures.

Plate 3.3 The effect of different lengths of time under anoxia followed by one week in sand, on the appearance of pea seedlings (20 - 24°C).

From left to right:

Rows I and II - 12, 10, 8, 6, 4, (2) h anoxia.

Rows III and IV - 24, 22, 20, 18, 16, (14) h anoxia.

CHAPTER 4

AEROBIC RESPIRATION

4.1 INTRODUCTION

Under natural conditions the whole plant is seldom subjected to conditions of total anoxia, but certain plant parts, frequently the roots, may be subjected to temporary or prolonged oxygen starvation. Short periods of flooding can prove lethal to many plants that normally grow in well-drained situations, including most crop species (Kramer and Jackson,1954; Bergman,1959) and Harris and Bavel (1957) concluded that "the most sensitive aspect of plant activity in regard to soil aeration" was root respiration. Flooding the soil displaces oxygen from the air spaces (Grable,1966; Ponnamperuma,1972), and therefore deficient aeration of the roots of plants not adapted to flooded conditions could result in root respiration and ultimately plant growth being impaired (Lemon and Wiegand,1962).

Aerobic respiration is a process involving the oxidative breakdown of organic substrate with the accompanying release of energy in the form of ATP (Beevers,1961), which is essential for the maintenance of a number of processes including ion uptake (Hoagland and Broyer,1936), translocation (Kursanov,1963) and membrane integrity (Hiatt and Lowe, 1967; Simon,1974). The enzyme systems involved in aerobic respiration are contained in mitochondria which are extremely sensitive to the absence of oxygen (Vartapetian et al.,1978) and observations of mitochondria from roots of rice (Vartapetian, Andreeva and Kozlova,1976), Triticale sp. (Oliveira,1977), cotton and pumpkin (Vartapetian et al., 1978) and tomato (Morisset,1978) have shown that changes in the ultrastructure of these organelles occur when the roots are subjected to

anoxia after a period of growth in air. Degradation of the mitochondria may occur if the period of anoxia is prolonged (Oliveira,1977).

The results in Chapter 3 showed that pea and rice seedlings differed in their tolerances of total anoxia. The decline and sudden drop in $\bar{R}s$ exhibited by pea and rice seedlings respectively (Figure 3.5) may reflect irrepairable damage to mitochondria sustained during the anaerobic treatments, resulting in the inability of seedlings to continue aerobic respiration and growth on return to air. Seedlings were therefore subjected to anoxia and the rate of oxygen uptake by the root tissue was measured either immediately after the anaerobic exposure, or after the whole seedling had experienced a 24 h recovery period in air. Initially the root tissue was not sterilised before the oxygen uptake determinations because of the possibility of further damaging the root, although later experiments were performed with surface sterilised tissue to reduce the bacterial contamination that accompanied damaged root systems after prolonged anaerobic exposures. Some seedlings were planted out in sand for one week after the anaerobic treatments and their $\bar{R}$ was calculated.

4.2 RESULTS

4.2.1 Oxygen uptake by pea root sections and detached, whole roots

A preliminary experiment was performed to assess the effect of age and /or distance from the root apex on the rate of oxygen uptake by excised, unsterilised pea root sections. Seedlings with ages ranging from 2-3 to 5-6 days old were examined following the method in Section 2.5.1, and the results are in Table 4.1. These results show that although there were no trends in any one group of sections relating to the age of the tissues, the apical 1 cm of each root had a higher rate of oxygen uptake than either of the other two root sections, the

oxygen uptake rates of which were similar.

Results in Figure 4.1a show the rates of oxygen uptake by detached, sterilised, whole root systems measured immediately after the seedling had been removed from the anaerobic environment, following the method in Section 2.5.2. As the length of the anaerobic exposure increased the resulting rate of oxygen uptake by the detached, whole roots on return to aerobic conditions gradually decreased, suggesting that damage to the aerobic respiration system had occurred, and that the amount of damage sustained increased with the length of the anaerobic treatment. However, after 24 h anoxia the rate of oxygen uptake was still more than half the control rate, a relatively high rate considering that the results in Chapter 3 had shown that the ability of pea seedlings to survive and grow after anoxia was lost after the whole seedling had been exposed to the anaerobic environment for more than 12 h. The apical 1 cm of the root has been shown to have a greater rate of oxygen uptake than other regions of the root, and therefore the effect of anoxia may be more marked, and hence better investigated, in the apical region of the root.

4.2.2 Oxygen uptake by unsterilised pea root tips

The rate of oxygen uptake by excised, unsterilised pea root tips was measured as described in Section 2.5.1. Figure 4.1b shows the rates of oxygen uptake by pea root tips immediately after the anaerobic exposure. There was a marked decrease in the rate of oxygen uptake as the length of the anaerobic exposure increased, and after 10 h anoxia the rate had declined to 11% of the control rate, although the rate then increased to a steady value after 18 h anoxia. Figure 4.2a shows the rate of oxygen uptake by pea root tips from seedlings that had remained in air for a 24 h recovery period after the anaerobic treatments. Seedlings which had been subjected to 10 h anoxia or less now exhibited oxygen

uptake rates between 57 and 100% of the control rate, whereas seedlings subjected to anoxia for 14 h or longer exhibited oxygen uptake rates lower than 57% of the control rate.

The solutions in which the seedlings had been exposed to anoxia were also assayed for K^+ (Section 2.4), since results in Chapter 3 had shown that only seedlings able to reabsorb most or all of the leaked K^+ appeared to recover from their anaerobic treatment. The results in Figure 4.2b show that the cut-off in the present experiment was between 10-12 h anoxia. Thus seedlings experiencing up to and including 10 h anoxia were not only able to increase their rate of oxygen uptake during the recovery period, but were also able to reabsorb most of the K^+ that had been lost from the roots during the anaerobic treatments.

4.2.3 Oxygen uptake by sterilised pea and rice (cv. Oeiras) root tips

The rate of oxygen uptake by excised, sterilised pea and rice root tips was measured as described in Section 2.5.1. Figure 4.3 shows the rates of oxygen uptake by pea root tips immediately after anoxia (a) and after the whole seedling had experienced a 24 h recovery period in air (b). As with unsterilised pea root tips (Figure 4.1b) the rate of oxygen uptake immediately after removal from the anaerobic environment decreased as the length of the anaerobic exposure increased, again suggesting that damage to the aerobic respiration system had occurred, and that it increased with the length of the anaerobic exposure. The visual symptoms of damage to pea seedlings in this experiment occurred between 6 and 8 h anoxia and the results in Figure 4.3b show that after 8 h anoxia the rate of oxygen uptake by these seedlings was much lower than the rate of oxygen uptake by seedlings that had been exposed to anoxia for 6 h or less. Furthermore, Figure 4.5a shows that seedlings planted out in sand after the anaerobic treatment failed to grow if the anaerobic exposure had been longer than 8 h.

Figure 4.4 shows the rates of oxygen uptake by rice root tips immediately after anoxia (a) and after the whole seedling had experienced a 24 h recovery period in air (b). Anaerobic exposures ranging from 2 to 12 h anoxia inclusive were not accompanied by successive decreases in the rate of oxygen uptake, and these seedlings exhibited a relatively steady rate of oxygen uptake suggesting that damage to the aerobic respiration system had not occurred during this time. However, between 12 and 14 h anoxia irreversible damage to the aerobic respiration system may have occurred, since none of the treatments from 14 to 24 h anoxia inclusive showed any oxygen uptake either on return to aerobic conditions or after the 24 h recovery period in air. After 14 h anoxia the $\bar{R}$ (see Figure 4.5b) fell sharply, probably because root growth had ceased as a result of damage to the aerobic respiration systems in the root. Continued growth of the shoot system, as previously discussed (see Section 3.3) could be responsible for further seedling growth after 14 h anoxia.

It is interesting to note that anoxia appears to have the same ultimate damaging effect on the aerobic respiration systems of both pea and rice seedlings, since the ability of the root tips to respire aerobically after an anaerobic exposure is closely followed by the ability of the seedlings to resume growth.

4.2.4 Bacterial contamination

Only in the experiments with unsterilised root tips (Sections 4.2.1 and 4.2.2) were relatively high rates of oxygen uptake obtained for the anaerobic exposures between 12 and 24 h, both in the immediate assay and after a 24 h recovery period in air. The solutions around the roots of the seedlings that recovered from their anaerobic treatments, i.e. the 0 to 10 h treatments inclusive, were clear whereas after the cut-off, .i.e after anaerobic exposures of 12 h or longer, the

solutions were cloudy. This cloudiness probably resulted from bacterial activity which would be enhanced by the loss of substances from the irreversibly damaged root tissue. Bacterial respiration could therefore account for a large proportion of the oxygen consumption obtained for these particular treatments, since the root tips were not washed or sterilised before the oxygen uptake determinations. Sterilised root tips exhibited oxygen uptake rates lower than unsterilised root tips and there was no increase in the rate of oxygen uptake from damaged seedlings (i.e. after the cut-off).

4.3 DISCUSSION

Initial experiments revealed that the apical 1 cm of the root had a higher rate of oxygen uptake than did other regions of the root, in agreement with the findings of other workers (Gregory and Woodford, 1939; Machlis,1944; Norris,1956; Beevers,1961; John et al.,1974). The effect of anoxia on the meristematic region of the root is of considerable importance since if the meristem is damaged, further new root growth from that meristem will be prevented (Morisset,1978). Working with excised, 8-10 week old tomato roots, Morisset (1978) showed that after 3 days anoxia previously growing meristems were irreversibly damaged. These present experiments show that after 6 and 12 h anoxia for pea and rice seedlings respectively, the ability of the root tips to resume oxygen uptake when removed from the anaerobic environment had been either greatly reduced (pea, Figure 4.3) or lost (rice, Figure 4.4), suggesting that irreversible damage to the aerobic respiration system had occurred during the anaerobic treatment. Other pea seedlings, subjected to anoxia at the same time as those used in these experiments, were used in growth experiments and the results in Figure 4.5 show that anaerobic exposures a few hours longer than those resulting in a loss

of much of the capacity for aerobic respiration (see Figure 4.3) markedly reduced seedling growth, suggesting that aerobic respiration is necessary for seedling recovery after anoxia.

Aerobic respiration produces 36 moles of ATP from 1 mole of glucose whereas in the absence of oxygen only 2 moles of ATP are available from the breakdown of 1 mole of glucose (Beevers,1961). If the capacity for aerobic respiration is severely damaged during the anaerobic treatment, the resulting energy supply on return to air may be insufficient to sustain all the ATP-requiring processes, culminating in cell and ultimately seedling death. Further evidence supporting the above suggestion is given by the results in Figure 4.2 which show that when the capacity for oxygen uptake, i.e. aerobic respiration, had been lost (after 10 h anoxia) the K^+ that had been lost from the roots during the anaerobic treatment was no longer reabsorbed. Potassium uptake, in common with the uptake of most cations and anions, is an active process requiring the ATP supplied by aerobic respiration (Hoagland and Broyer, 1936), and it would be expected to cease when aerobic respiration was prevented. Aerobic respiration was necessary for K^+ uptake and seedling recovery after anoxia and therefore it is interesting to note that Mullett and Considine (1980), investigating the relationship between K^+ loss and reuptake with the physiological condition of some leguminous seeds, found that radicle extension occurred only where a net uptake of K^+ had occurred.

Oliveira (1977) found that periods of anoxia greater than 24 h were increasingly deleterious to the metabolism and structure of Triticale sp. root cells, but if the period of anoxia was less than 24 h, the roots were able to recover. Similarly, Coulomb and Coulomb (1972b, cited by Oliveira,1977) found that Cucurbita pepo plants exposed to anoxia recovered when transferred to an aerated environment if the anaerobic

period had not been prolonged. In these present experiments with pea and rice seedlings the capacity for aerobic respiration by pea root tips was progressively decreased as the period of anoxia increased (Figure 4.3) and after 6 h anoxia none of the seedlings recovered, whereas with rice root tips the capacity for aerobic respiration was maintained after up to and including 12 h anoxia (Figure 4.4) and some growth of the seedlings was observed after all the anaerobic exposures. Thus seedlings recover from exposure to anoxia if the exposures are less than a certain limit which appears to be species-dependent. If this limit is exceeded, irreversible damage to the aerobic respiration system occurs resulting in the inability of seedlings to resume oxygen uptake and growth on return to aerobic conditions. The loss of aerobic respiratory activity may be connected with the degradation of mitochondria, the sites of aerobic respiration in cells, during anoxia (Oliveira, 1977).

Ueda and Tsuji (1971) suggested that the conformational changes of mitochondria in rice coleoptiles during germination in nitrogen gas were caused by shortage of energy for the maintenance of normal conformation, and Luzikov, Zubatov and Rainina (1973) found that oxidative phosphorylation was necessary for the maintenance of mitochondrial activity *in vivo*. Thus it is possible that the inhibition of oxidative phosphorylation and ATP production in mitochondria during anoxia damages these organelles so that on return to aerobic conditions the original rates of ATP production cannot be attained. Alternatively, the accumulation of ethanol within the cell could ultimately have the same effect through its fluidising effect on the mitochondrial membrane (Crawford, 1977).

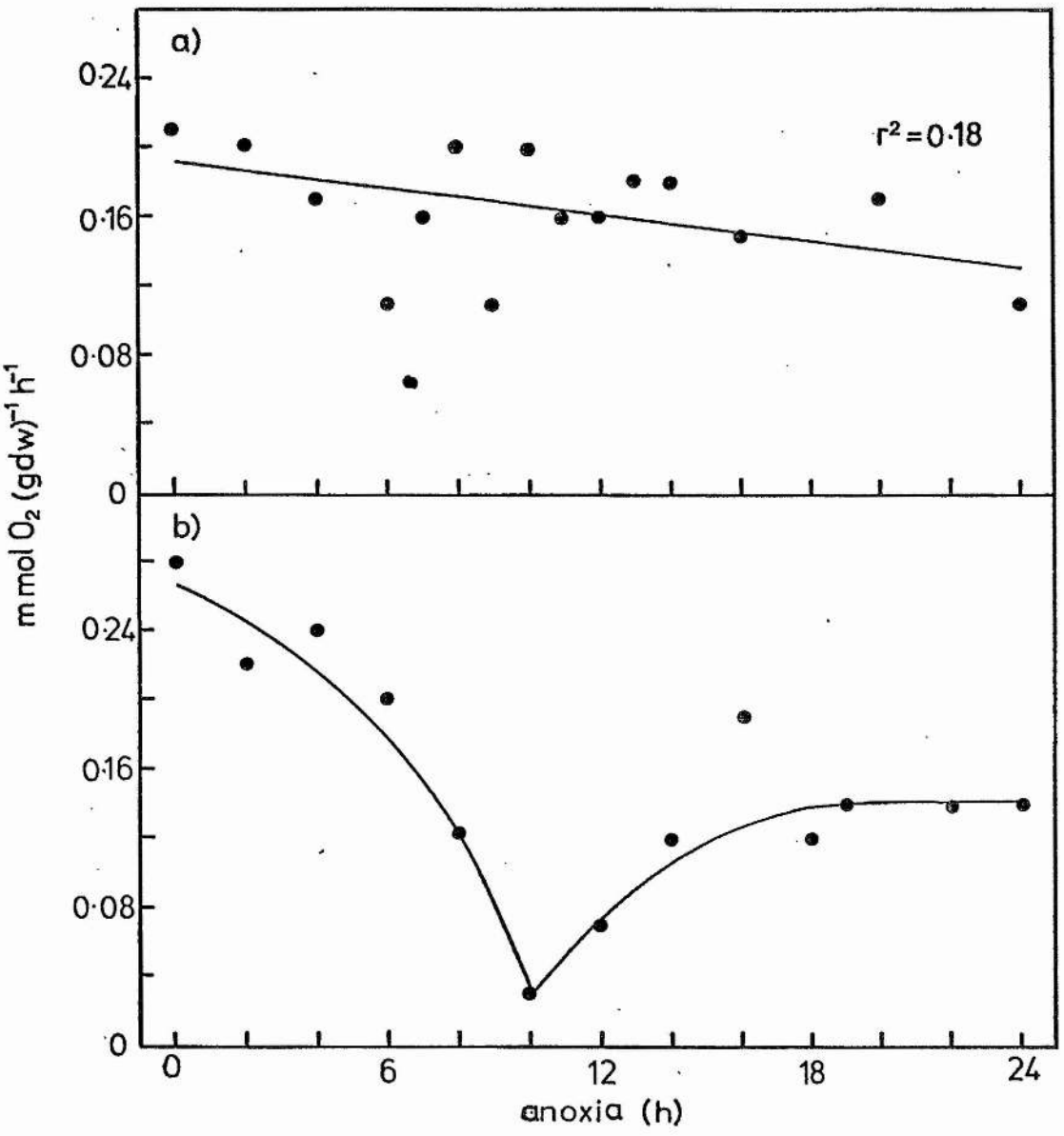


Figure 4.1 The effect of different lengths of time under anoxia on the immediate oxygen uptake rates of pea roots.

(a) Excised, sterilised whole root systems.

(b) Excised, unsterilised root tips.

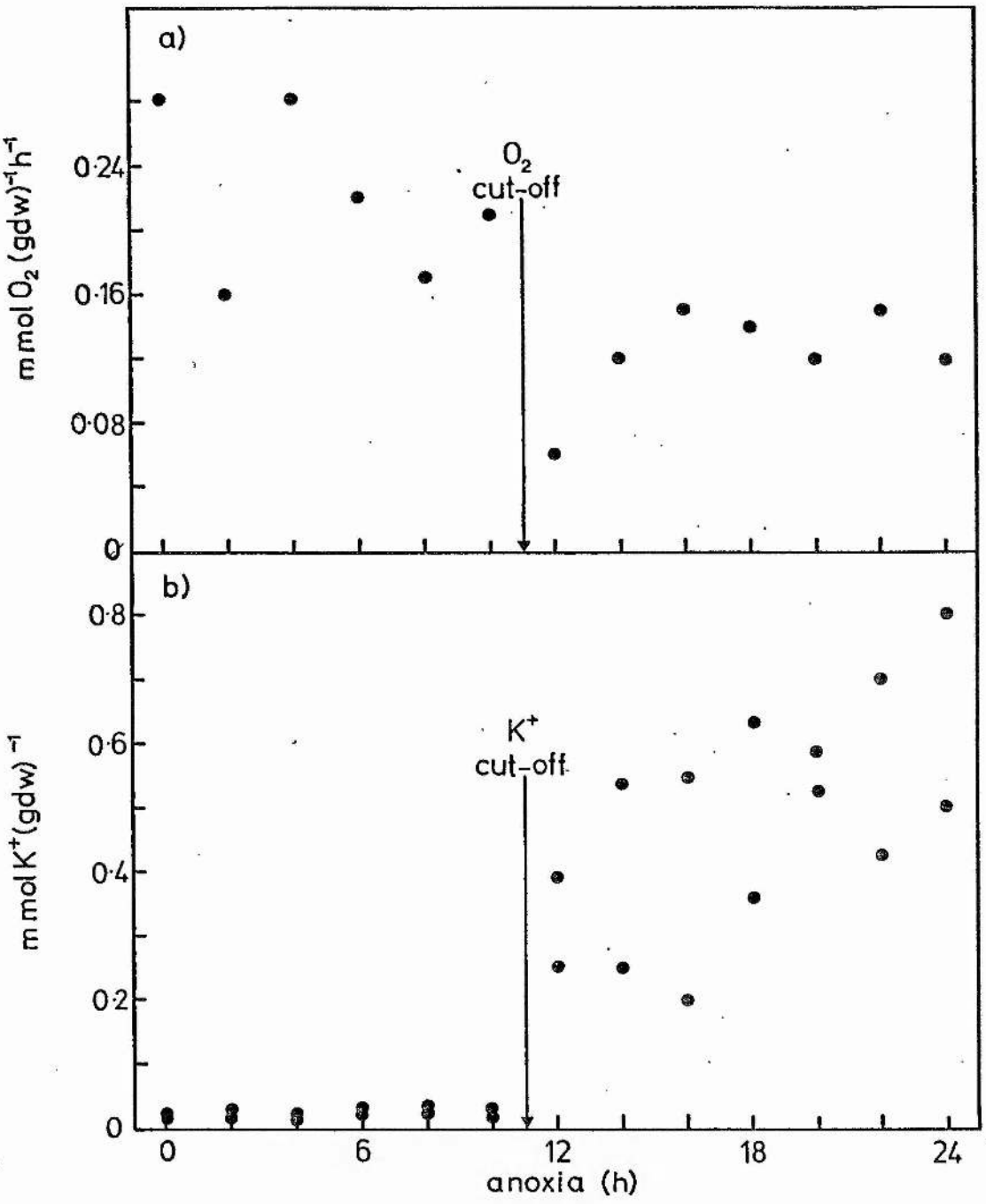


Figure 4.2 The effect of different lengths of time under anoxia followed by a 24 h recovery period in air on: (a) The oxygen uptake rates of excised, unsterilised pea root tips, and (b) the K^+ remaining in the solution around the roots of intact pea seedlings.

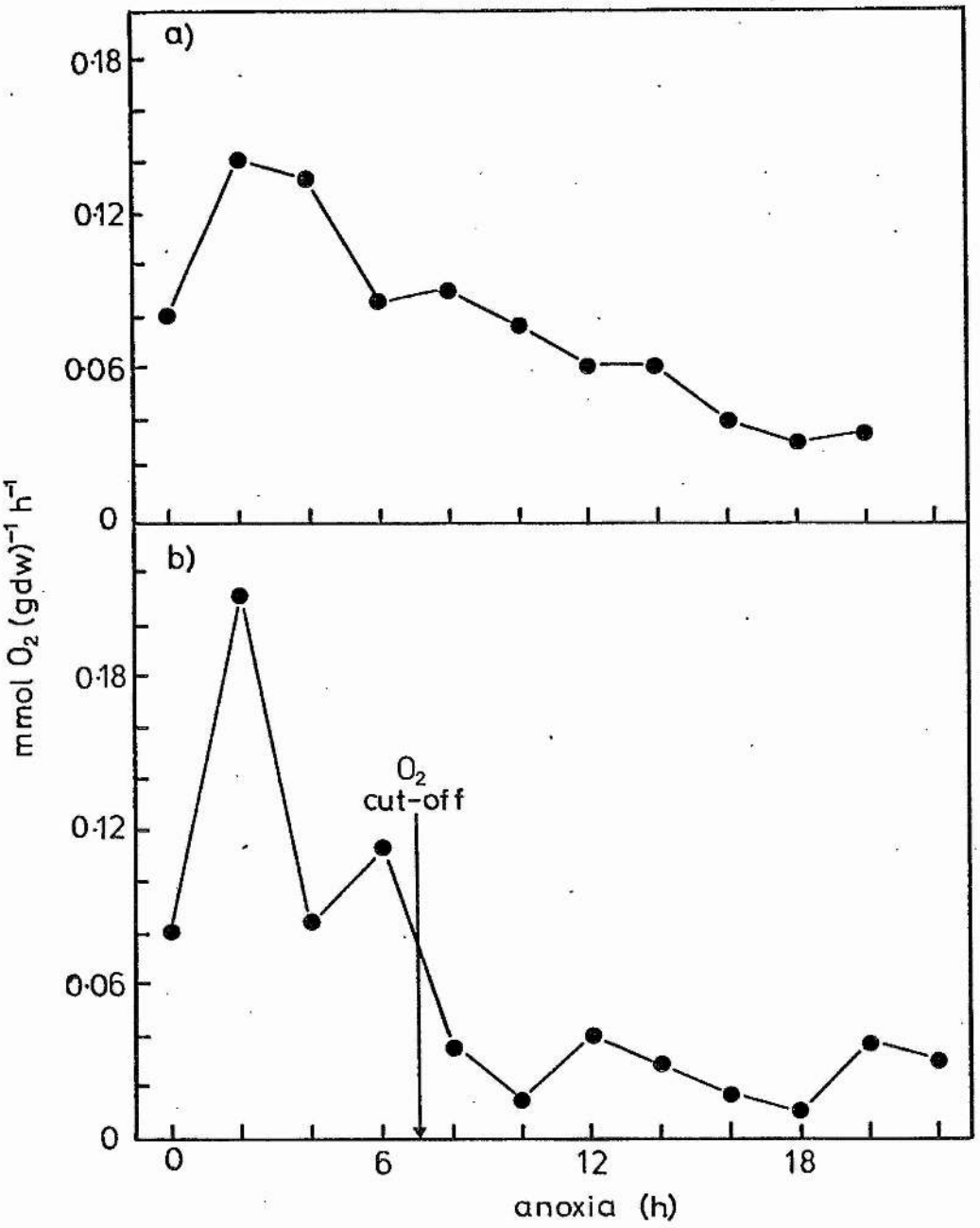


Figure 4.3 The effect of different lengths of time under anoxia on the oxygen uptake rates of excised, sterilised pea root tips (20°C).

(a) Immediately after anoxia.

(b) After a 24 h recovery period in air.

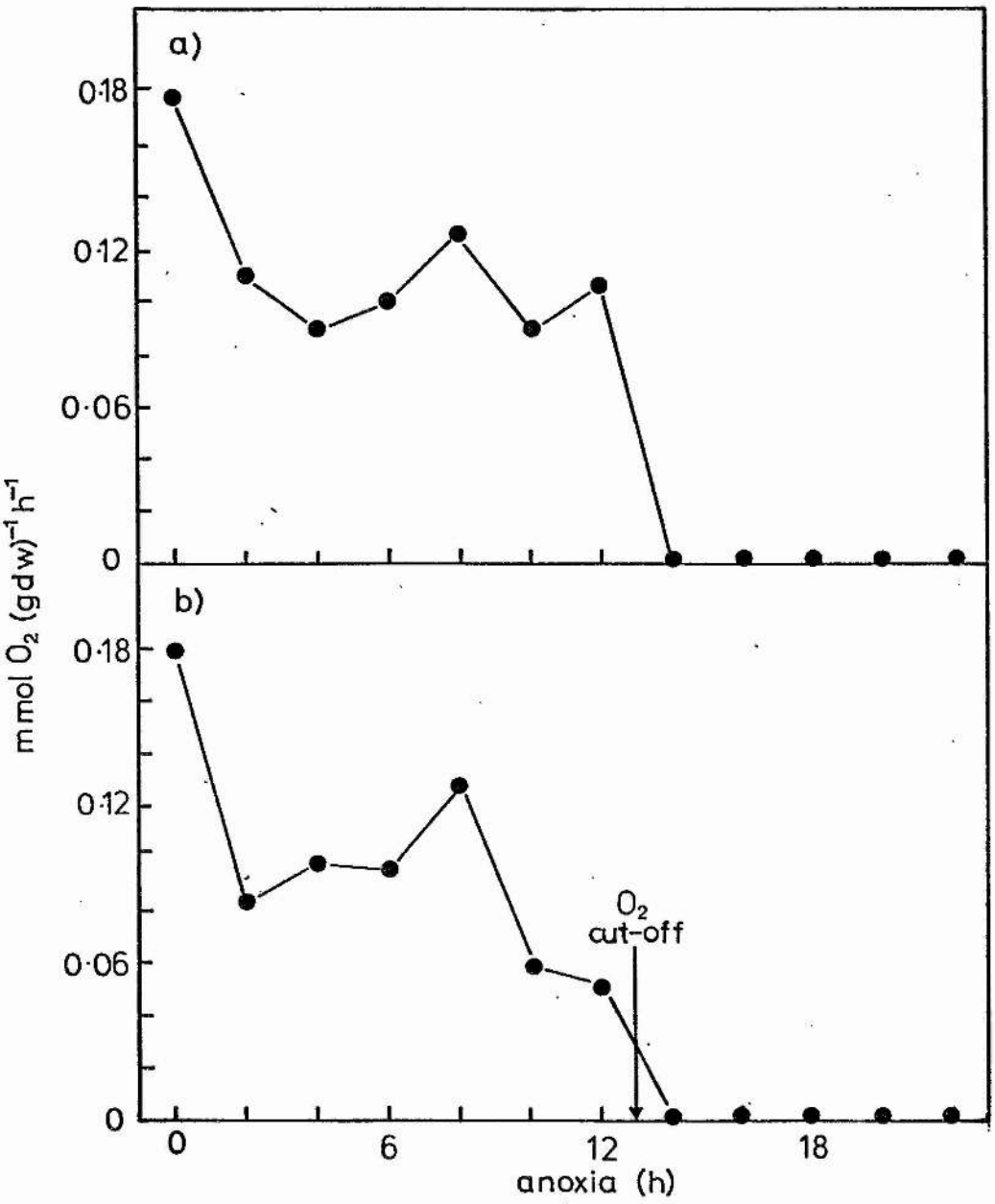


Figure 4.4 The effect of different lengths of time under anoxia on the oxygen uptake rates of excised, sterilised rice root tips (25°C).

(a) Immediately after anoxia.

(b) After a 24 h recovery period in air.

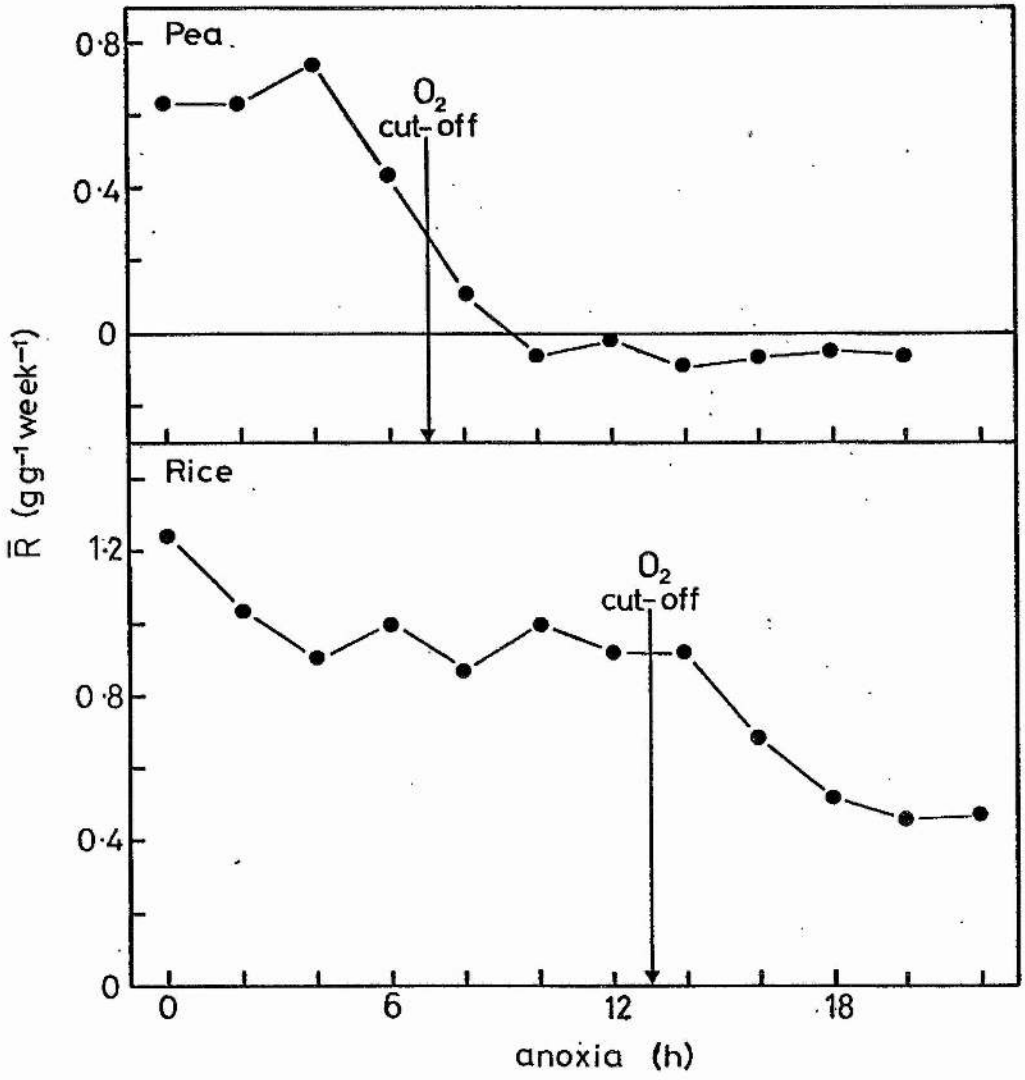


Figure 4.5 The effect of different lengths of time under anoxia on the subsequent mean relative growth rates, $\bar{R}$, of pea (20°C) and rice (25°C) seedlings after one week in sand. (n = 2).

Table 4.1

The rate of oxygen uptake (mmol gdw^{-1} h^{-1}) at 20°C by excised, unsterilised pea root sections of different ages, as a function of the distance from the root apex.

	mmol O_2 $(gdw)^{-1}$ h^{-1}		
	Distance from apex (cm)		
Age (days)	0 - 1	1 - 2	2 - 3
2 - 3	0.211	0.206	0.162
3 - 4	0.404	0.180	0.147
4 - 5	0.300	0.199	0.248
5 - 6	0.345	0.191	0.191
mean ± s.e.	0.315 ± 0.032	0.194 ± 0.004	0.186 ± 0.017

CHAPTER 5

ETHANOL ACCUMULATION

5.1 INTRODUCTION

Ethanol accumulates in some plant tissues under anaerobic conditions (Kenefick,1962; Fulton and Erickson,1964; Grineva,1964; Leblova et al.,1969; Bolton and Erickson,1970; Andrews,1977; Chirkova,1978) and this has been regarded by some workers as harmful to the plant (Fulton and Erickson,1964; Andrews,1977; Chirkova,1978; Pradet and Bomsel,1978). Ethanol is thought to have its toxic effect by attacking and fluidising cell membranes (Kiyosawa,1975; Nandini-Kishore et al.,1979) and intolerance of some plants to flooding has been attributed to toxic concentrations of ethanol accumulating within the tissues (Fulton and Erickson, 1964; Crawford,1967; Crawford and Baines,1977), a situation intensified in the roots of many flood-intolerant species by the acceleration of glycolysis under anoxia (Crawford,1966;1967;1978). However, there are very few reports in the literature on the concentrations of ethanol accumulating in plant tissues under anaerobic conditions. Fulton and Erickson (1964) found that ethanol supplied to tomato plants in nutrient solution in concentrations similar to those found in the xylem exudate of flooded plants (4.3-8.7 mmol l^{-1}) were toxic, and they suggested that the permanent injury to tomato plants caused by short term flooding may have resulted from the toxic effects of endogenous ethanol. The experiments of Barclay and Crawford (1981) also implicated ethanol accumulation as a cause of seedling death under anoxia, since pea seedling survival at different temperatures was always associated with an internal ethanol concentration of less than 60 mmol l^{-1}. However, there is no conclusive proof that the quantities of ethanol accumulating in the

flooded roots of flood-intolerant plants are either toxic to the plant or the cause of flooding injury in these species.

Many investigations into the toxicity of ethanol on plant tissues have involved applying it externally (Beletskaya,1977; Chirkova,1978; Morisset,1978), but although Beletskaya (1977) found that 217 mmol l^{-1} ethanol reduced the viability of germinated winter wheat and rye, Morisset (1978) found this concentration to be non-toxic to excised tomato roots. Chirkova (1978) used ethanol concentrations of 10^{-3} and 10^{-2} mol l^{-1} but found no signs of injury in willow (flood-tolerant) and poplar (flood-intolerant) plants exposed to these concentrations for three weeks, and it is possible that under aerobic conditions some of the externally applied ethanol may be metabolised by the roots (Cossins, 1978). However, App and Meiss (1958), who germinated and grew rice in various concentrations of ethanol for one week, found that concentrations greater than 10^{-1} mol l^{-1} were toxic to the seedlings.

In the absence of oxygen, ethanol may accumulate to toxic levels within tissues, especially in flooded roots where there may be insufficient internal ventilation to maintain aerobic respiration in all the regions of the root. It is interesting to note that rice, a flood-tolerant plant producing ethanol and exhibiting an accelerated glycolysis under anoxia (Taylor,1942), not only has an efficient internal ventilating system (Barber et al.,1962; Armstrong,1978) but also excretes much of the ethanol produced (Bertani et al.,1980) thereby preventing ethanol accumulation within the tissues. Other flood-tolerant species have been shown to remove ethanol from the tissues, for example ethanol is lost from willow plants via the lenticels (Chirkova and Gutman,1972) and 85% of the ethanol produced by Echinochloa crus-galli seedlings under anoxia was found in the external medium (Rumpho and Kennedy,1981).

Pea and rice, cv. Oeiras, seedlings were subjected to anoxia and

the ethanol accumulating around the roots was measured. Pea seedlings were also subjected to anoxia in sodium bisulphite ($NaHSO_3$) solutions in an attempt to reduce ethanol production, and hence accumulation, through the reaction of the bisulphite moiety with acetaldehyde, the precursor of ethanol (see Section 2.6.3). Pea root ethanol content and the possibility of ethanol loss via the leaves of pea seedlings during anoxia were also examined. In addition, the sensitivities of pea and rice, cv. Oeiras, seedlings to a range of ethanol concentrations under aerobic conditions were compared, and the effect of ethanol on pea seedling survival under anoxia was also investigated.

5.2 RESULTS

5.2.1 Ethanol loss from roots

Pea and rice seedlings were subjected to different lengths of time under aerobic and anaerobic conditions and the ethanol content of the solution around the roots was determined enzymatically immediately after these exposures (Sections 2.6.1 and 2.6.2). Since previous experiments (Section 3.2) have shown that damage to pea seedlings occurred after less than 12 h anoxia at 20°C, ethanol exudation by pea roots was measured after anaerobic exposures of up to and including 12 h only. These results are in Figure 5.1a, and they show that under anaerobic conditions ethanol accumulated in the solution around the roots, whereas under aerobic conditions it did not. Similar results were obtained with rice seedlings at 20°C (Figure 5.1b). At 25°C more ethanol accumulated around rice roots than at 20°C (Figure 5.1b), presumably because at the higher temperature the metabolic rate, and hence ethanol production, were increased. Pea roots lost more ethanol during the first 12 h anoxia than did rice roots at either 20 or 25°C - after 10 h anoxia pea roots had accumulated 0.66 mmol ethanol per gramme dry weight of root, an

amount not reached by rice roots until after 20 h anoxia at the same temperature, or after 12 h anoxia at 25°C. Thus the tolerance limits of pea and rice seedlings under anoxia may be associated with the production and accumulation of ethanol.

5.2.2 Ethanol production by pea roots

The patterns of ethanol exudation observed may reflect patterns of ethanol production and accumulation internally, or alternatively all the ethanol produced may be exuded. The quantities of ethanol accumulating in the roots and in the solutions around the roots were determined enzymatically (Sections 2.6.1 and 2.6.2) after seedlings had been subjected to anoxia in distilled water, and these results are in Figure 5.2a. The K^+ content of the solutions were also measured (Section 2.4) and the time of cut-off determined by this method was used as an indicator of irreversible damage to the seedlings (Figure 5.2b). These results show that the time of maximum internal and external ethanol accumulations coincided with the time of the cut-off, although this does not necessarily mean that ethanol was the cause.

Seedlings were also subjected to anoxia in sodium bisulphite ($NaHSO_3$) solutions in an attempt to reduce ethanol production and thus delay the onset of damage during anoxia. Figure 5.3 shows the effect of $NaHSO_3$ on the ethanol content of the solutions around pea roots immediately after the anaerobic treatment. Dilute concentrations (10^{-4} and 10^{-3} mol l^{-1}) had no effect on the ethanol content of the solutions, which were similar to the water controls, but at a concentration of 10^{-2} mol l^{-1}, $NaHSO_3$ markedly reduced the quantity of ethanol found around the roots. Potassium ions could not be measured accurately in $NaHSO_3$ solutions because Na^+ interferred with the absorption spectrum of K^+, but since the time of cut-off determined by K^+ analysis after a 24 h recovery period has been shown to coincide with the observed symptoms of

damage, such observations were used instead. However, although 10^{-2} mol l^{-1} $NaHSO_3$ reduced the ethanol content of the solution around the roots, all the $NaHSO_3$ treatments and the water controls exhibited symptoms of damage at the same time. A further experiment, the results of which are in Figure 5.4, showed that although the presence of 10^{-2} mol l^{-1} $NaHSO_3$ during anoxia again reduced the ethanol content of the solution around the roots, the root ethanol content in the two treatments were similar, and furthermore the times of cut-off were the same. The markedly lower quantities of external ethanol in the 10^{-2} mol l^{-1} $NaHSO_3$ treatments may have resulted from the inhibition of bacterial ethanol production by this concentration of $NaHSO_3$, since unsterilised root tissue was used.

5.2.3 Prevention of transpiration

Pea seedlings were prevented from transpiring during the anaerobic treatments by either removing the shoot systems, or spraying them with S600, an antitranspirant which formed a plastic coating over the aerial parts (Section 2.7.1). However, these two treatments differed in that removal of the shoot system also prevented translocation of substances between the leaves and the roots. Figure 5.5 shows that the time of cut-off, as determined by the analysis of net K^+ after a 24 h recovery period in air, occurred 2 h earlier when seedlings were prevented from transpiring during the anaerobic treatment than it did when seedlings were allowed to transpire. This could reflect a more rapid accumulation of a toxic substance which is normally removed by transpiration during anoxia. Therefore a further experiment was performed where pea seedlings were subjected to anoxia and then sprayed with S600 on removal from the anaerobic environment, i.e. they were allowed to transpire during the anaerobic treatment. Seedlings treated in this way exhibited symptoms of damage at the same time as the transpiring controls, verifying the

above suggestion that a toxic substance is transpired during anoxia.

5.2.4 Summary of the above results

Unsterilised roots of whole pea and rice seedlings produced more ethanol under anoxia than under aerobic conditions, although some of the ethanol production was attributed to bacteria. The accumulation of ethanol in the solution around pea roots was not reflected in the root ethanol content, although ethanol may have reached toxic concentations at localised sites within the cells, the cut-off occurring when a critical number of these sites had been damaged. Such localised accumulations would not be detected in estimates of total root ethanol content. Further evidence for the accumulation of a toxic substance within the roots during anoxia comes from the transpiration experiments where pea seedlings that were prevented from transpiring during the anaerobic treatment cut-off earlier than transpiring control seedlings. The prevention of transpiration during anoxia would result in the more rapid accumulation of the toxin, and therefore these seedlings would be expected to be damaged earlier than the control seedlings.

5.2.5 Effect of exogenous ethanol on seedling growth

Pea and rice, cv. Oeiras, seedlings were subjected to 1, 3 or 5 days in ethanol solutions in air and their growth was followed by measuring changes in fresh weight (Sections 2.6.4 and 2.6.5 respectively). Figures 5.6a and 5.7a show the effect of ethanol on the mean relative growth rate, $\bar{R}$, of pea and rice seedlings respectively. The final weights of the 1 day treatments were taken 7 days after the initial weighings whereas the final weights of the 5 day treatments were taken 11 days after the initial weighings. The rate of seedling growth depends on, among other factors, the age of the seedling and so marked differences in the $\bar{R}$ of the three controls were apparent. Therefore Figures 5.6b and 5.7b show the $\bar{R}$ of pea and rice seedlings expressed as a per-

centage of the appropriate water control so that the different treatments may be compared.

To determine which ethanol treatments were significantly different from the water controls, t-tests were performed on the $\bar{R}$ data. These results are in Table 5.1 and they show that the $\bar{R}$ of both pea and rice seedlings was unaffected by concentrations of ethanol up to 10^{-1} mol l^{-1}, which is equivalent to 500 µmol ethanol in a 5 ml tube, until after 3 days exposure. After 5 days in ethanol solutions growth of rice seedlings was significantly reduced in 10^{-2} mol l^{-1} ethanol, but growth of pea seedlings was not significantly reduced until a concentration of 10^{-1} mol l^{-1} was reached. Rice seedlings were exposed to more ethanol per gramme dry weight of root tissue than were pea seedlings - there was usually more pea root tissue per seedling, and therefore per tube, than rice root tissue - which may account for this difference, although this seems unlikely because the solutions were renewed every 12 h to maintain the ethanol levels.

Plate 5.1 shows the appearance of pea seedlings after one week in ethanol solutions. Concentrations of 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} mol l^{-1} ethanol appeared to have no effect on seedling growth and these treatments resembled the water control. In 10^{-1} mol l^{-1} ethanol there was some development but no growth of lateral roots, although the shoot system appeared to have been unharmed, whereas in 1.0 mol l^{-1} (1M) ethanol there was no development of lateral roots and the shoot systems were wilted and yellow.

5.2.6 Effect of exogenous ethanol during anoxia

A concentration of 0.1 mol l^{-1} ethanol has been shown to have no damaging effect on pea seedlings exposed to this concentration for up to 3 days in air, but a 0.3 mol l^{-1} solution damaged the growth of pea seedlings after 24 h (Table 5.1). Pea seedlings were subjected to anoxia

in solutions of 0.1 and 0.3 mol l^{-1} ethanol for 1 to 12 h inclusive, and the K^+ content of the solution was measured after the seedlings had experienced a 24 h recovery period in air (Section 2.4). These results are in Figure 5.8, and each graph is the mean result of two separate experiments in which the water controls cut-off at the same time. In 0.1 mol l^{-1} ethanol the cut-off is shown to occur later than the control (a and b), although in one experiment the ethanol treatment and the control cut-off at the same time. However, the fact that the cut-off did not occur earlier in 0.1 mol l^{-1} ethanol than it did in water, suggests that exogenous ethanol at this concentration is not toxic to the seedlings during anoxia.

The cut-off occurred earlier in 0.3 mol l^{-1} ethanol than it did in water on two occasions, as the mean result in Figure 5.8 (c and d) indicates, possibly because this concentration of ethanol <u>per se</u> was sufficient to denature the plasmalemma of the root cells and irreversibly damage the seedlings. Neither 0.1 nor 0.3 mol l^{-1} ethanol caused any leakage of K^+ from the roots when seedlings were subjected to these concentrations in air for similar lengths of time, i.e. up to 12 h, possibly because the seedlings were able to metabolise some of the ethanol during these limited exposures. However, prolonged exposures to 0.1 and 0.3 mol l^{-1} ethanol in air, where the solutions were renewed every 12 h, have been shown to damage pea seedling growth (Table 5.1). Under these circumstances ethanol may again damage seedling growth through its detrimental effect on the plasmalemma of the root cells.

5.3 DISCUSSION

Results in Chapter 3 revealed that rice seedlings could endure anoxia for longer periods than pea seedlings, and therefore it is interesting to note that ethanol accumulated less rapidly around rice roots

than around pea roots during anoxia (Figure 5.1). Moreover, rice seedlings have been shown to tolerate anoxia longer at 20°C than at 25°C (Section 3.2.3) and Figure 5.1 shows that ethanol accumulated around rice roots more slowly at 20°C than at 25°C. Ethanol loss from anaerobic roots has also been reported for pea (Aubertin, Rickman and Letey,1966), tomato (Bolton and Erickson,1970), wheat (Chirkova,1978), rice (Chirkova, 1978; Bertani et al.,1980) and barnyard grass (Rumpho and Kennedy,1981). In these present experiments seedlings with unsterilised roots were used and therefore some of the ethanol accumulating around the roots during the anaerobic treatments could have been of bacterial origin. However, this situation resembles that of flooded roots in the field, where the activities of microorganisms in waterlogged soils may lead to the formation of lower aliphatic alcohols which may accumulate to concentrations that are harmful to plant growth (Wang and Chuang,1967). These authors reported that a concentration of 0.4 mol l^{-1} ethanol damaged shoot growth of rice seedlings. In these present experiments the quantity of ethanol in solution reached 1.11 mmol $(gdw)^{-1}$ around pea roots after 12 h anoxia, and 1.80 mmol $(gdw)^{-1}$ around rice roots after 22 h anoxia, equivalent to ethanol concentrations of 1.86 and 1.80 mmol l^{-1} respectively. These concentrations, measured a few hours after the known tolerance limits of pea and rice seedlings under anoxia, are remarkably similar and may therefore indicate that a toxic concentration of exogenous ethanol has been exceeded.

The concentrations of ethanol found around the roots during anoxia are relatively low compared with those necessary to significantly reduce seedling growth under aerobic conditions (Table 5.1). However, in air there is the possibility that the exogenous ethanol may be metabolised by the seedlings (Cossins and Beevers,1963; Chirkova,1978). Chirkova (1975) found that the roots of willow, a flood-tolerant plant,

remained viable in 10^{-1} mol l^{-1} ethanol for 6 days whereas the roots of poplar, a flood-intolerant plant, were killed by this concentration after 2 days, and she later concluded (Chirkova,1978) that the resistance of plants to oxygen deficiency correlated with their resistance to higher concentrations of the glycolytic end-products. However, the results in Table 5.1 are at variance with the above hypothesis, since after 5 days in 10^{-2} mol l^{-1} ethanol growth of rice, a flood-tolerant plant, was significantly reduced whereas growth of pea, a flood-intolerant plant, was unaffected. Ingram (1976) found that cells of Escherichia coli K12, changed their fatty acid composition in the presence of alcohol, and Thomas et al. (1978) found that in Saccharomyces cerevisiae the tolerance of exogenous ethanol was related to the lipid composition of the plasmalemma. It is therefore possible that the plasmalemma in root cells of pea and rice seedlings were of different compositions, which affected their permeability properties and hence the rate of diffusion of ethanol into the roots.

The growth of pea seedlings in air was not significantly reduced after 5 days in a concentration of 10^{-1} mol l^{-1} ethanol, approximately fifty times stronger than the concentration measured around the roots during anoxia. Furthermore, pea seedlings subjected to anoxia in this concentration of ethanol were not damaged earlier than the control seedlings in water (Figure 5.8), suggesting that the external concentration of ethanol was not the cause of the cut-off. Fermenting yeast cells also produce and excrete ethanol, the production of which ceases and cell death occurs once a certain external concentration has been reached (Gray,1941). However, if active yeast cells are exposed to these apparently lethal concentrations of ethanol the cells are not killed and it is thought that the internally produced ethanol is considerably more toxic than that applied externally (Nagodawithana and Steinkrauss,1976).

A similar situation may exist in higher plants and although measurement of the ethanol content of pea roots produced conflicting results (Figures 5.2 and 5.4), the possibility of toxic accumulations of ethanol at specific sites within the cell cannot be ruled out.

Results in Section 4.2.3 showed that irreversible damage to the aerobic respiration systems of pea and rice seedlings occurred following anaerobic exposures in excess of 6 and 12 h respectively (Figure 4.5). Mitochondria are membrane-bound structures and the sites of aerobic respiration in cells, and since ethanol is thought to have its toxic effect by attacking and fluidising cell membranes (Kiyosawa,1975; Nandini-Kishore et al.,1979), its accumulation in the cytoplasm near mitochondria during anoxia may result in the inactivation of the mitochondria once a tolerable concentration has been exceeded (Crawford,1977). Nobel (1973) found that mitochondria and chloroplasts from different tissues varied in their permeability properties for non-electrolytes, including alcohols, and therefore it is possible that rice seedlings were more tolerant of endogenous ethanol than pea seedlings. This together with the suggestion that rice root cells may be more permeable to ethanol than pea root cells may explain how the accumulation of harmful quantities of ethanol within rice seedlings during anoxia is delayed.

In addition to ethanol loss from anaerobic roots by diffusion into the surrounding medium, some ethanol may be removed from the roots in the transpiration stream and lost via the leaves. Bolton and Erickson (1970) found that of the ethanol formed in flooded tomato roots, one fifth was eliminated through excretion and one twentieth by transpiration, and Kenefick (1962) reported that transpiration was one of the means of ethanol removal from the roots of anaerobically treated sugar beets. If a toxic product of anaerobic metabolism was being removed from the root tissues by transpiring leaves, then the prevention of transpiration

during anoxia would be expected to result in a more rapid accumulation of the toxic substance in the roots, and hence earlier death of the seedlings. Results in Figure 5.5 show that seedlings treated in this manner during the anaerobic exposures were damaged earlier than transpiring control seedlings, a result in agreement with the above hypothesis. Similarly, Fulton and Erickson (1964) found higher ethanol concentrations in xylem exudates from decapitated rather than intact tomato plants, and they attributed the difference to the elimination of ethanol via the foliage of intact plants.

Thus the accumulation of a toxic product of anaerobic metabolism, assumed but not proved to be ethanol, may cause the pea seedling cut-off during anoxia through its damaging effect on the mitochondria. A similar situation may exist in rice seedlings during anoxia.

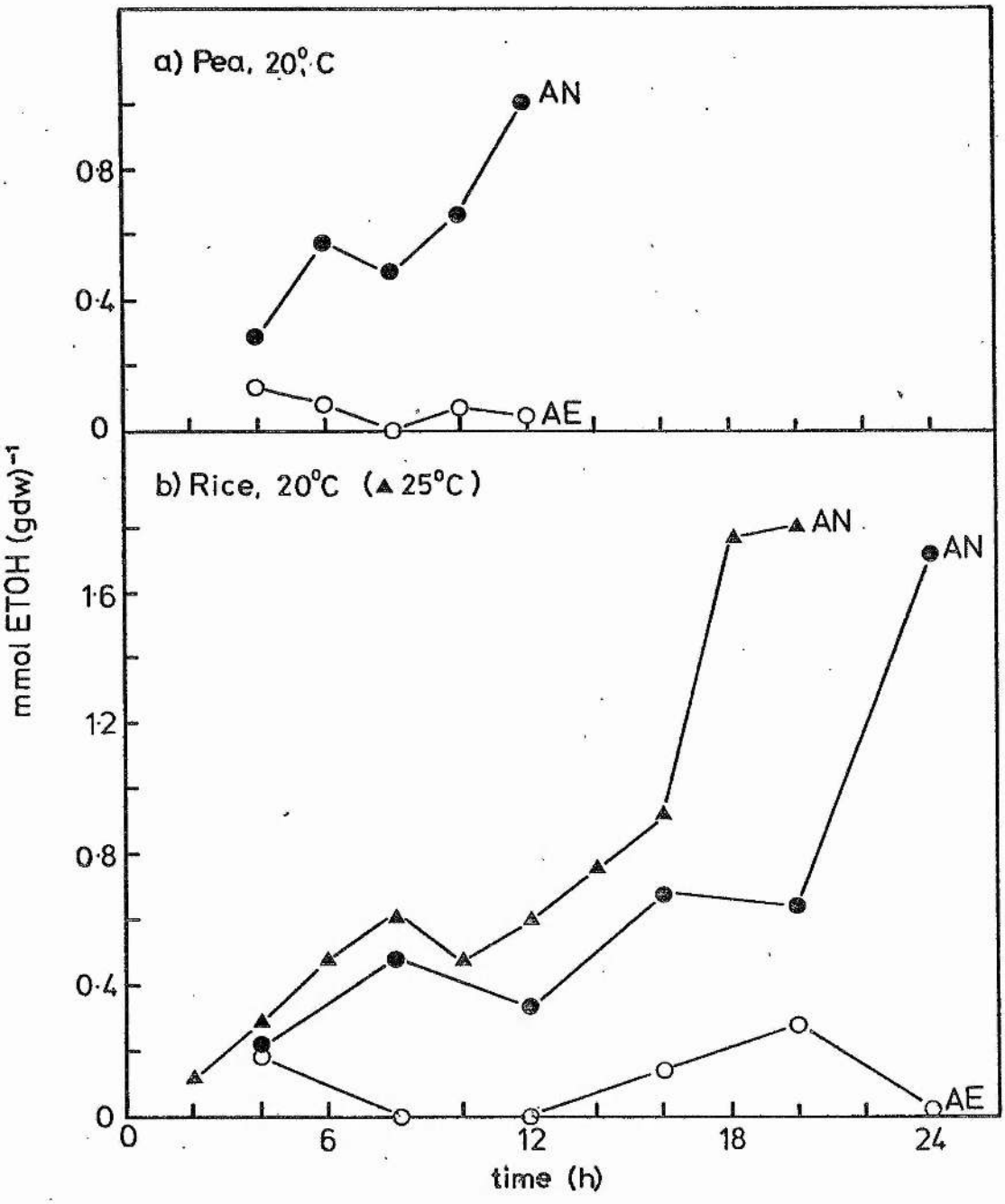


Figure 5.1 Ethanol accumulation in the solution around the roots of intact seedlings under aerobic (AE) and anaerobic (AN) conditions.

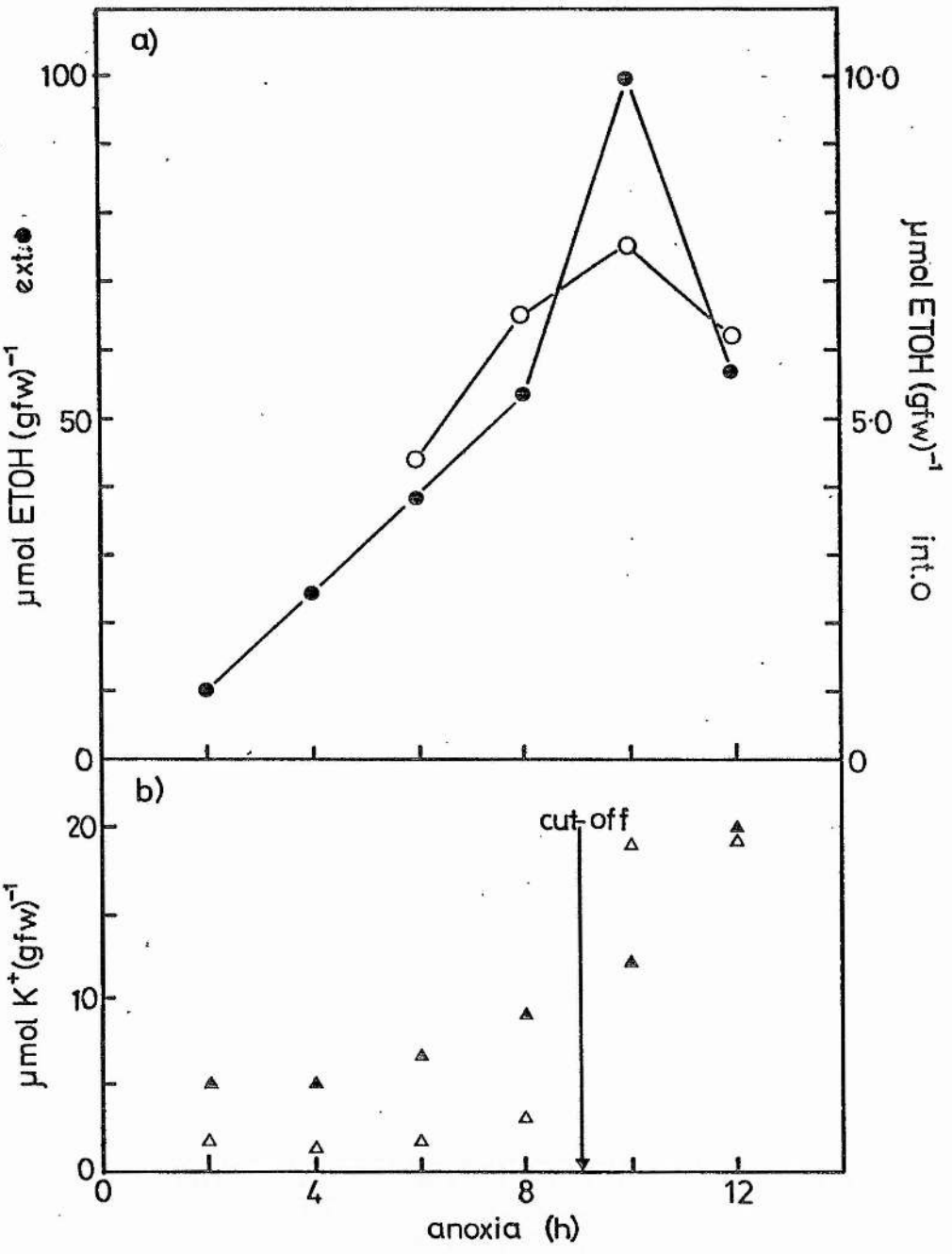


Figure 5.2 The effect of different lengths of time under anoxia on: (a) Ethanol accumulation in the roots, and in the solution around the roots, of intact pea seedlings, and (b) the K^+ remaining in the solution around these roots immediately after anoxia (▲) and after the recovery period in air (△).

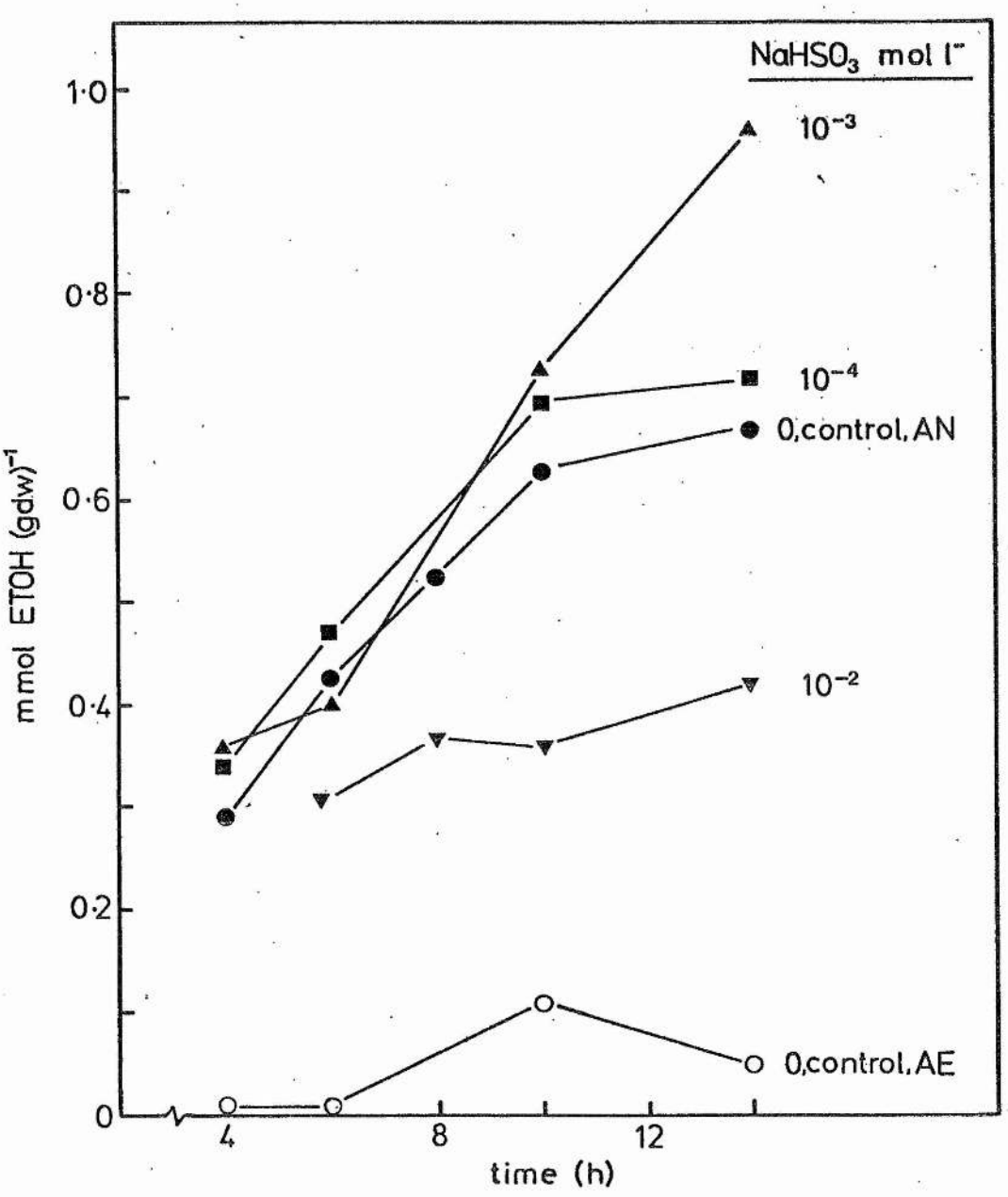


Figure 5.3 The effect of different concentrations of $NaHSO_3$ on ethanol accumulation in the solution around the roots of intact pea seedlings during anoxia, compared with water controls both in air (AE) and under anoxia (AN).

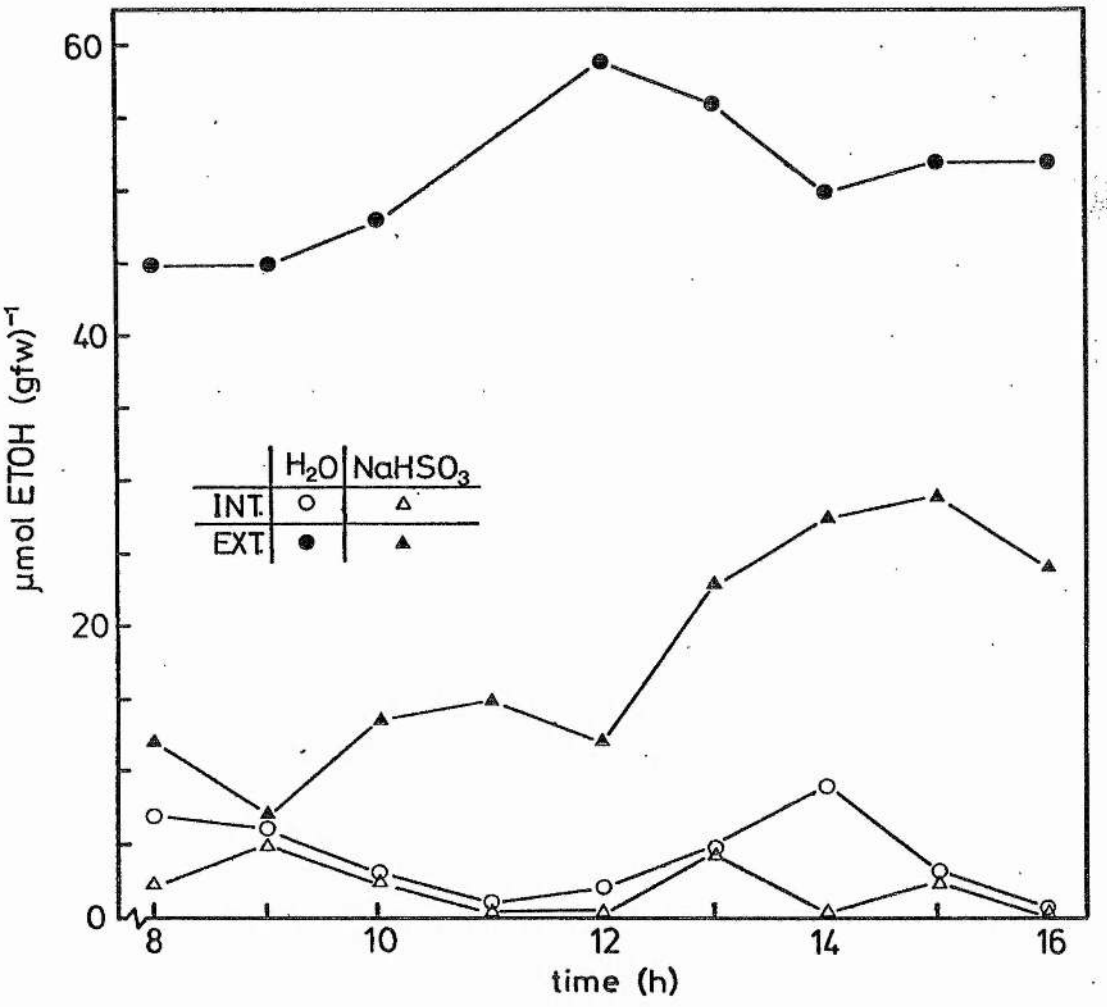


Figure 5.4 The effect of different lengths of time under anoxia on ethanol accumulation in the roots (INT.), and in the solutions around the roots (EXT.), of intact pea seedlings in water and in 10^{-2} mol l^{-1} $NaHSO_3$.

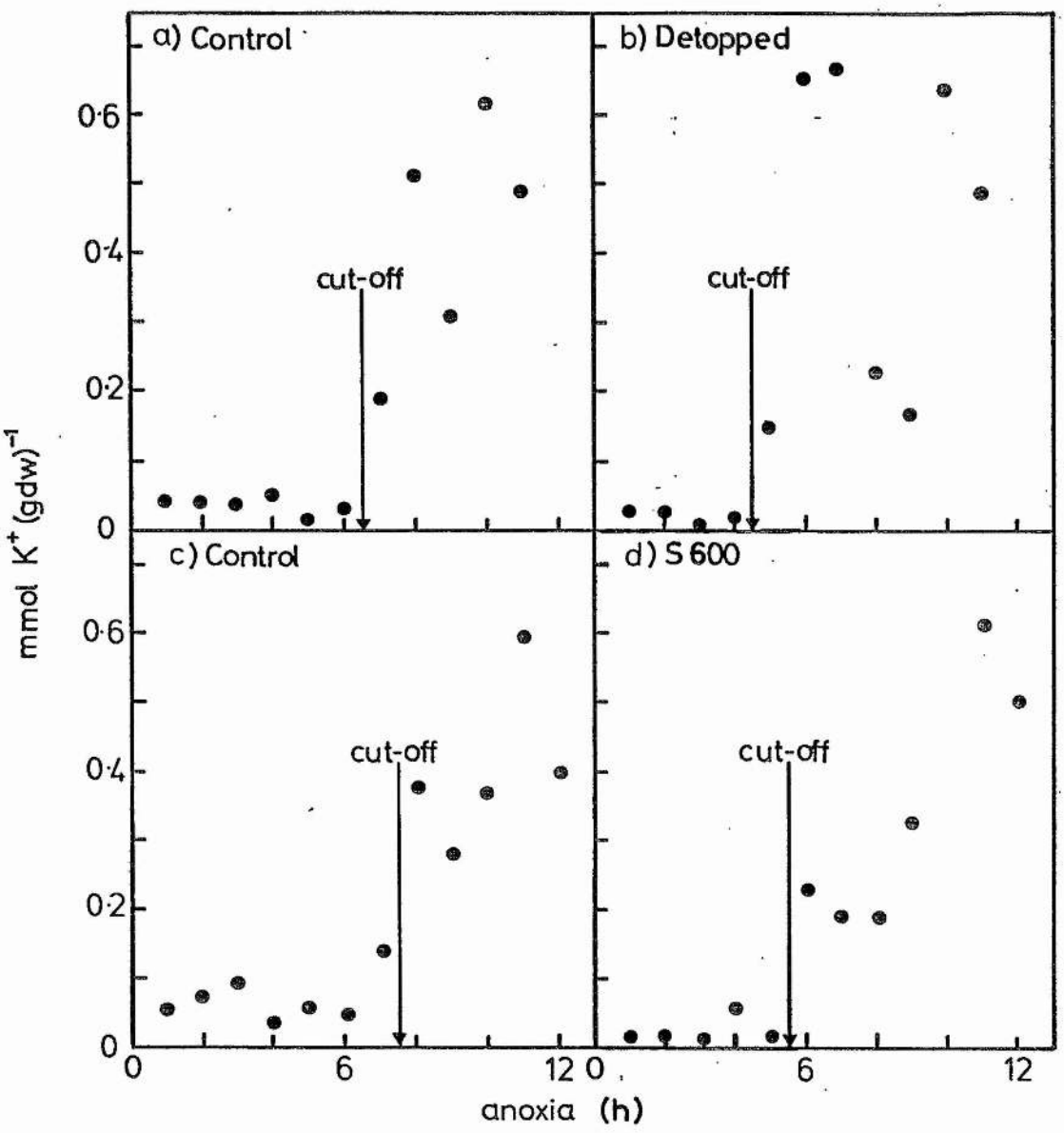


Figure 5.5 The effect of preventing transpiration (b and d) on the time of cut-off by pea seedlings compared with transpiring controls (a and c).

Figure 5.6 The effect of exposing the roots of intact pea seedlings to different concentrations of ethanol for 1 (●), 3 (▲) or 5 (■) days, on the subsequent mean relative growth rate, $\bar{R}$, (20°C). (n = 6).

(a) Actual $\bar{R}$

(b) $\bar{R}$ expressed as a percentage of the appropriate water control.

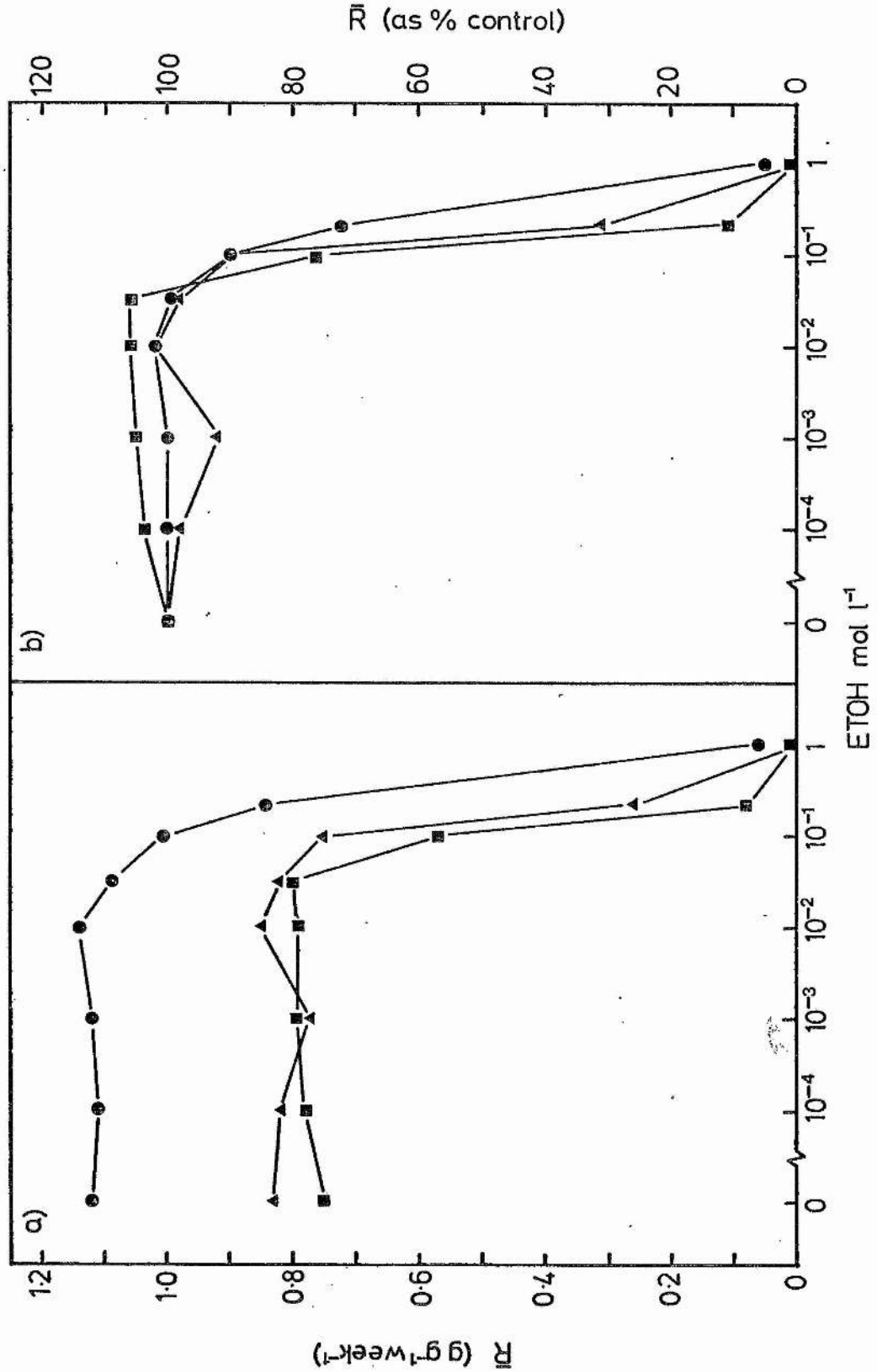
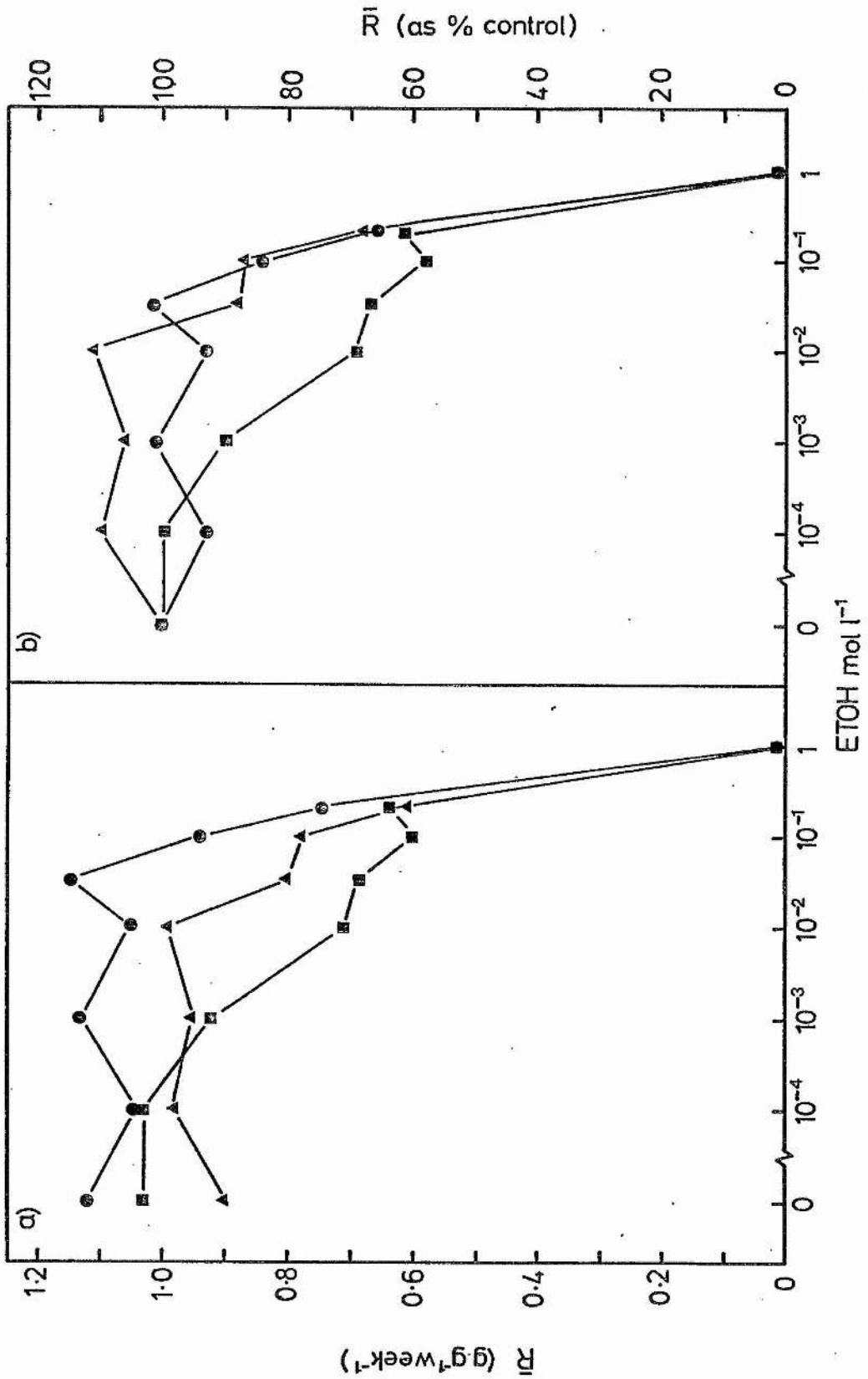

R̄ (as % control)
120
100
80
60
40
20
0
b)
a)
R̄ (g g⁻¹ week⁻¹)
1·2
1·0
0·8
0·6
0·4
0·2
0
0
10⁻⁴
10⁻³
10⁻²
10⁻¹
1
ETOH mol l⁻¹

Figure 5.7 The effect of exposing the roots of intact rice seedlings to different concentrations of ethanol for 1 (●), 3 (▲) or 5 (■) days, on the subsequent mean relative growth rate, $\bar{R}$, (25°C). (n = 6).

(a) Actual $\bar{R}$.

(b) $\bar{R}$ expressed as a percentage of the appropriate water control.


R (g.g⁻¹week⁻¹)
1·2
1·0
0·8
0·6
0·4
0·2
0
a)
b)
R̄ (as % control)
120
100
80
60
40
20
0
0
10⁻⁴
10⁻³
10⁻²
10⁻¹
1
ETOH mol l⁻¹

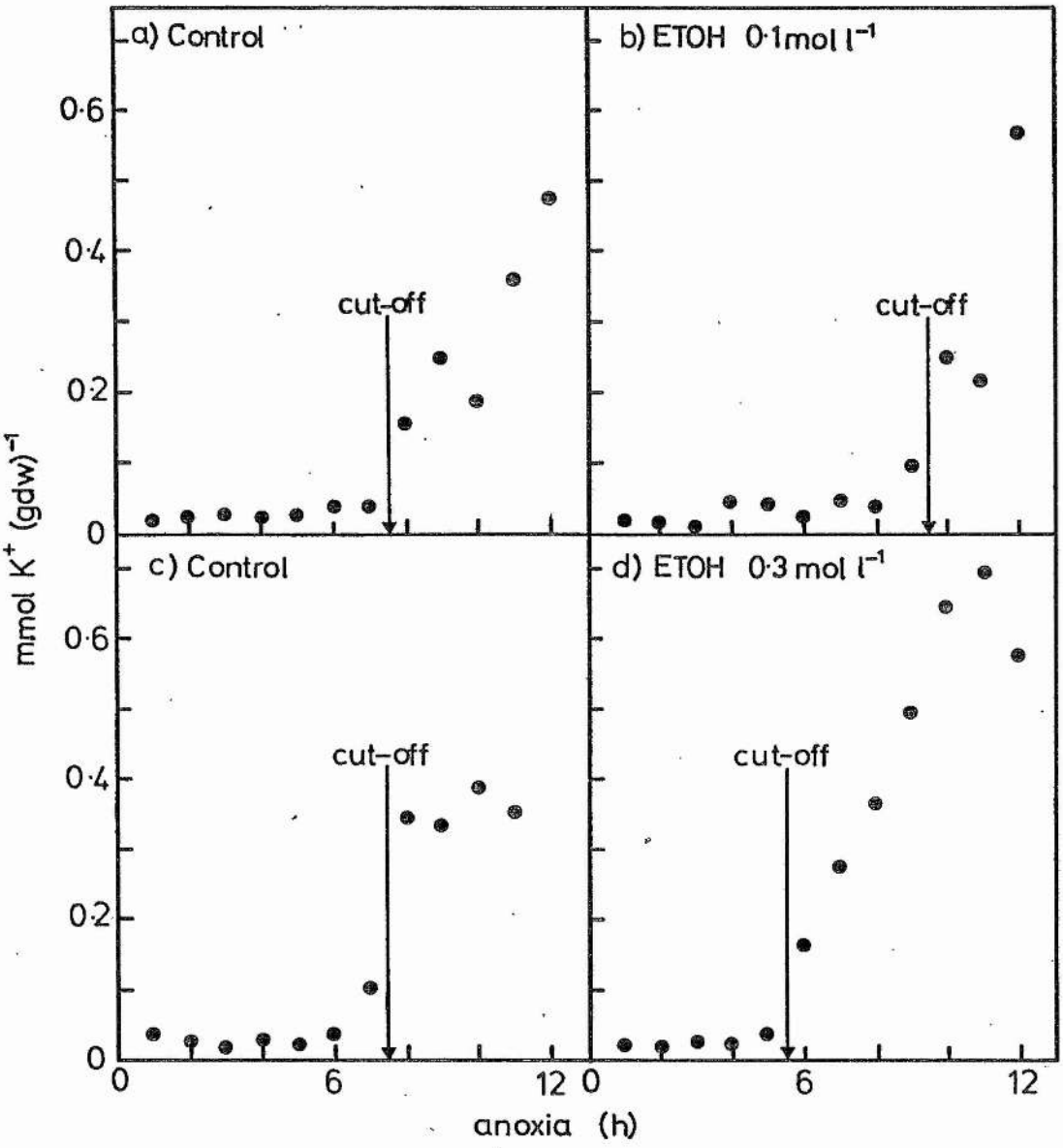


Figure 5.8 The effect of two different ethanol concentrations (b and d) on the time of cut-off by pea seedlings compared with water controls (a and c).

Table 5.1

The mean relative growth rate, $\bar{R}$, of pea and rice (cv. Oeiras) seedlings after 1, 3 or 5 days exposure of the roots to water or different concentrations of ethanol; t-tests (n = 6) compare the ethanol treatments with the relevant water controls. Based on data presented in Figures 5.6a and 5.7a.

		Concentration of ethanol (mol l^{-1})					
Species	Exposure (days)	10^{-4}	10^{-3}	10^{-2}	5×10^{-2}	10^{-1}	3×10^{-1}
a) Pea	1	n.s	n.s	n.s	n.s	n.s	***
	3	n.s	n.s	n.s	n.s	n.s	***
	5	n.s	n.s	n.s	n.s	*	***
b) Rice	1	n.s	n.s	n.s	n.s	n.s	**
	3	n.s	n.s	$*^1$	n.s	n.s	***
	5	n.s	n.s	*	**	***	**

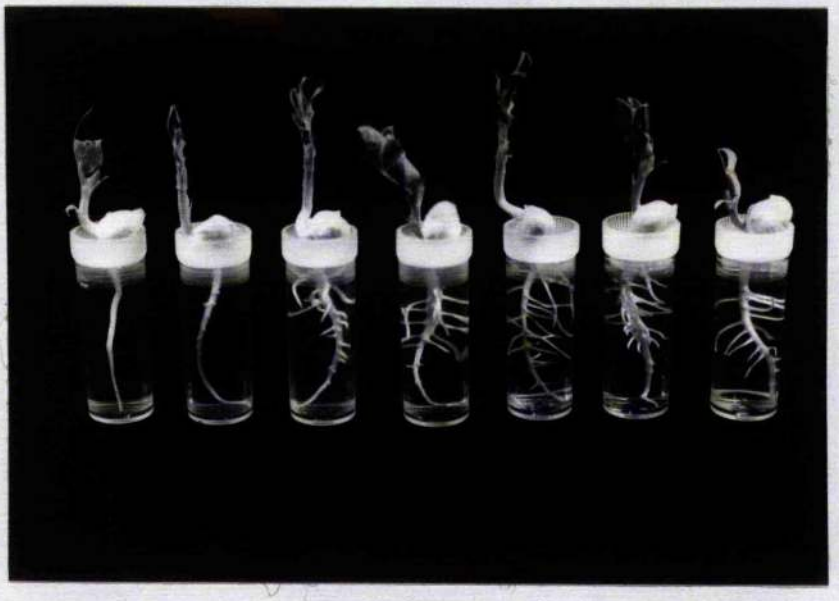
n.s - no significant difference at the 5% level

* - significant decrease at the 5% level

** - significant decrease at the 1% level

*** - significant decrease at the 0.1% level

$*^1$ - significant increase at the 5% level



1 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 0

Concentration of ethanol (mol l^{-1}) Control

Plate 5.1 The effect on the appearance of pea seedlings, of exposing the roots to different concentrations of ethanol for one week.

CHAPTER 6

CARBOHYDRATE AVAILABILITY

6.1 INTRODUCTION

Glycolysis operates in the presence and absence of oxygen but its rate is dependent upon oxygen availability (Beevers,1961). In many tissues an increase in the rate of glycolysis occurs when the tissue is deprived of oxygen (Beevers,1961; Crawford,1978) and this causes an increase in the rate of production and accumulation of its end-product, lactate or ethanol, which some workers (Fulton and Erickson,1964; Andrews, 1977; Chirkova,1978; Pradet and Bomsel,1978) consider harmful to the plant. However, the increase in glycolytic rate also results in an increased rate of production of ATP which other workers (Grineva,1964; Rumpho and Kennedy,1981) consider an adaptation to anaerobic conditions because more energy is made available during the period of anoxia.

An increase in the rate of glycolysis must also be accompanied by an increase in the rate of consumption of substrates, particularly glucose, the hexose at the starting point of the glycolytic sequence, and this in turn requires a more rapid breakdown of organic compounds to supply the glucose. Translocation, in common with other ATP-requiring processes, may be reduced or completely inhibited during anoxia (Curtis, 1929, cited by Bergman,1959; Kursanov,1963) and therefore there is the possibility that glycolysis may be restricted or even cease through lack of substrate if the tissues do not possess sufficient reserves of organic compounds. Vartapetian et al. (1976) suggested that the capacity of the rice coleoptile to grow during anoxia was a result of the seedlings' ability to transport organic compounds from the grain to the coleoptile for utilisation in glycolysis, and they advanced the hypothesis

(Vartapetian et al.,1977) that the immediate factor responsible for the injury of the root cells of mesophytes during anoxia may be carbon starvation. Further experiments with excised roots and glucose solutions led them to conclude (Vartapetian et al.,1978) that the ultrastructure of rice roots does not have a higher tolerance of anoxia than the roots of pumpkin, a flood-intolerant plant, and that carbon starvation is the immediate cause of root cell destruction under prolonged anoxia. Unpublished data of Gaynard and Armstrong, Webb and Armstrong (cited by Sanderson and Armstrong,1980) showed that rice and pea roots in glucose media regrew after a 24 h exposure of the whole plant to anoxia, whereas roots without added glucose died.

The experiments in this chapter were designed to investigate the importance of carbohydrate reserves for pea seedling survival during and after anoxia. Seedlings with different carbohydrate contents - i.e. light-grown and dark-grown seedlings, both with and without cotyledons - were used. In some experiments 2% glucose was supplied to the roots during the course of the experiment and its effect on K^+ loss and seedling survival were examined. Ethanol accumulation in the solution around the roots was also investigated.

6.2 RESULTS

6.2.1 Effect of sugar on pea seedling survival under anoxia

Seedlings were subjected to anoxia in distilled water, 2% glucose or 2% sucrose solutions (Section 2.3) and the K^+ content of the solution was determined (Section 2.4). The results in Figure 6.1 show that seedlings subjected to anoxia in water lost more K^+ than seedlings subjected to anoxia in either 2% glucose or 2% sucrose solutions, and there are two possible reasons for this. Firstly, the roots in sugar solutions may have been less severly damaged than roots in water, hence smaller

quantities of K^+ were lost. Alternatively, the rate of diffusion of K^+ into water may have been greater than its rate of diffusion into the sugar solutions. After a 24 h recovery period in air the time of cut-off, as determined by measurement of the net K^+ in solution, was between 6-7 h anoxia for the control seedlings in water, but was unclear for the sugar treatments (Figure 6.2), since the quantities of K^+ remaining in these solutions were relatively low for all anaerobic exposures. Nevertheless, visible symptoms of damage were apparent in all three treatments, although it is interesting to note that damage was observed in control seedlings 1 h earlier than it was in the sugar treatments, i.e. the presence of sugar during anoxia delayed the onset of damage. A further experiment was performed to determine if the presence of glucose during anoxia also enhanced the subsequent performance of seedlings planted out in sand (method in Section 2.2.3). Observations again showed that seedlings that had been subjected to anoxia in 2% glucose solutions exhibited symptoms of damage 1 h later than the control seedlings. However, when these seedlings were planted out in sand their performances were similar. Figure 6.3 shows the fresh weight changes of these seedlings after one week in sand, and Figure 6.4 shows the same data expressed as the mean relative growth rate, $\bar{R}$. A paired t-test on the mean data from Figure 6.3 revealed that the water and glucose treatments were not significantly different at the 5% level ($t = 1.866$, $df = 12$), i.e. the presence of 2% glucose during anoxia, although beneficial in the short term, did not enhance the subsequent growth or survival of the seedlings.

6.2.2 Effect of carbohydrate availability during anoxia

Light-grown and dark-grown pea seedlings, some of which had had their cotyledons removed immediately before the experiment (Section 2.7.2), were subjected to anoxia in water or 2% glucose solutions (Section 2.3)

and the K^+ content of the solutions determined after a 24 h recovery period in air (Section 2.4). The cotyledons are storage organs containing reserves of carbohydrate which are metabolised during the growth of the seedling. These organs were removed in order to reduce the quantity of carbohydrate available to the seedling during anoxia. The results in Figure 6.5 show that seedlings without cotyledons exhibited an earlier cut-off than intact seedlings, which suggests that some translocation of organic material from the cotyledons occurred in intact seedlings during anoxia. Thus the earlier cut-off by seedlings without cotyledons may have been caused by a shortage of organic compounds. To test this hypothesis seedlings without cotyledons were subjected to anoxia in 2% glucose solutions in an attempt to replace the carbohydrate reserves removed with the cotyledons. The results of this experiment are in Figure 6.6 and they show that the cut-off was delayed when these seedlings were subjected to anoxia in 2% glucose solutions compared to when they were subjected to anoxia in water.

Exogenous glucose has also been shown to delay the cut-off by whole seedlings, which suggests that the endogenous supply of organic compounds may be insufficient to maintain metabolism during prolonged anoxia. However, the exogenous glucose merely delayed the symptoms of damage. It is unlikely that the quantity of exogenous glucose was limiting since 2% glucose delayed the cut-off by light-grown seedlings without cotyledons for 2 h (Figure 6.6), but delayed the cut-off by whole seedlings for only 1 h (observation), although whole seedlings had an additional source of carbohydrate in the cotyledons. This result suggests that a shortage of carbohydrate was not the cause of the cut-off when whole seedlings were subjected to anoxia in 2% glucose solutions.

6.2.3 Ethanol accumulation

Ethanol is the end-product of glucose metabolism in pea seedlings

during anoxia, and it is possible that in a situation where glycolytic substrate is not limiting metabolism, ethanol may accumulate to toxic levels within the tissues. Dark-grown and light-grown pea seedlings, some of which had had their cotyledons removed immediately before the experiment (Section 2.7.2), were subjected to anoxia in water or 2% glucose solutions (Section 2.3). The ethanol content of the solutions was determined enzymatically immediately after the anaerobic treatment (Sections 2.6.1 and 2.6.2) and the results are in Table 6.1. It is interesting to note the time at which the maximum quantity of ethanol had accumulated for each treatment - whole light-grown and dark-grown seedlings continued to accumulate ethanol throughout the course of the experiment, whereas seedlings without cotyledons reached their maximum ethanol accumulation after 8 h anoxia. This suggests that in seedlings without cotyledons glycolytic substrate was exhausted after 8 h anoxia. Further evidence for this comes from the result that light-grown and dark-grown seedlings without cotyledons, but subjected to anoxia in 2% glucose solutions, continued to accumulate ethanol for longer periods than similar seedlings subjected to anoxia in water, presumably because the exogenous glucose was being metabolised. The cut-off by seedlings without cotyledons, but subjected to anoxia in 2% glucose solutions, may have been caused by either a shortage of glycolytic substrate or the accumulation of toxic quantities of ethanol. However, when whole seedlings were subjected to anoxia in 2% glucose solutions, a situation where glycolytic substrate was not considered limiting, seedling death may have been caused by ethanol. Unfortunately ethanol accumulation around the roots of whole seedlings subjected to anoxia in 2% glucose solutions was not investigated.

6.3 DISCUSSION

Light-grown pea seedlings that were subjected to anoxia in water

showed symptoms of damage earlier than seedlings that were subjected to anoxia in 2% solutions of glucose and sucrose. This suggested that the seedlings in water may have been experiencing a shortage of glycolytic substrate during the anaerobic treatment. Such a shortage could have resulted from either inadequate carbohydrate reserves in the seedling, or inefficient breakdown and translocation of the reserves during anoxia. Seedlings without cotyledons exhibited earlier cut-offs than similar seedlings in 2% glucose (Figure 6.6) or whole seedlings in water (Figure 6.5). This suggests, contrary to reports in the literature (Kursanov, 1963; Vartapetian et al.,1978), that some translocation of sugar from the cotyledons did occur in whole seedlings during anoxia. However, that this endogenous supply of sugar was insufficient to maintain the required rate of glycolysis in whole seedlings during anoxia, is evidenced by the observation that 2% glucose also delayed the cut-off by whole, light-grown seedlings. Similarly, Vartapetian et al. (1978) found that the destructive changes observed in excised pupmkin and rice roots, after 5 and 7 h anoxia respectively, could be delayed until after 96 h anoxia if the roots were supplied with exogenous glucose (0.5 to 1.0% solutions).

The 2% glucose solution contained sufficient glucose to delay the cut-off by light-grown seedlings without cotyledons for 2 h, and therefore a similar solution was expected to the delay the cut-off by whole, light-grown seedlings for at least 2 h, since these seedlings also possessed further organic reserves in the cotyledons. However, 2% glucose solutions merely delayed the cut-off by whole, light-grown seedlings for 1 h, suggesting that in this case something other than a sugar shortage was the cause of the cut-off. If there was a shortage of glycolytic substrate, the rate of glycolysis, and hence the rate of ATP production, would be reduced and under these circumstances seedling death under

anoxia may result from insufficient ATP for the maintenance of cellular processes. However, when glycolytic substrate is not limiting, glycolysis would be expected to proceed at its maximum anaerobic rate, and although there is the possibility that ethanol may accumulate to levels that are harmful to the seedling, there is also the possibility that the maximum rate of ATP production is still insufficient to maintain cellular processes during prolonged anoxia. Similar conclusions were reached by Vartapetian et al. (1978) for excised rice roots, since increasing the glucose concentration from 1 to 3% did not further enhance the resistance of the root cells to anoxia.

Measurement of ethanol accumulation around the roots during anoxia showed that seedlings without cotyledons ceased accumulating ethanol earlier than either whole seedlings or seedlings without cotyledons that were in 2% glucose solutions (Table 6.1). This would be expected because seedlings without cotyledons would exhaust their supplies of glycolytic substrate earlier than whole seedlings or those with an exogenous glucose supply. However, the cause of death of whole, light-grown seedlings that were subjected to anoxia in 2% glucose solutions still remains unclear; seedling death under these conditions may have resulted from the accumulation of harmful quantities of ethanol and /or the failure of cellular processes, including the maintenance of mitochondrial integrity, because the rate of ATP production during anoxia could not satisfy the demand.

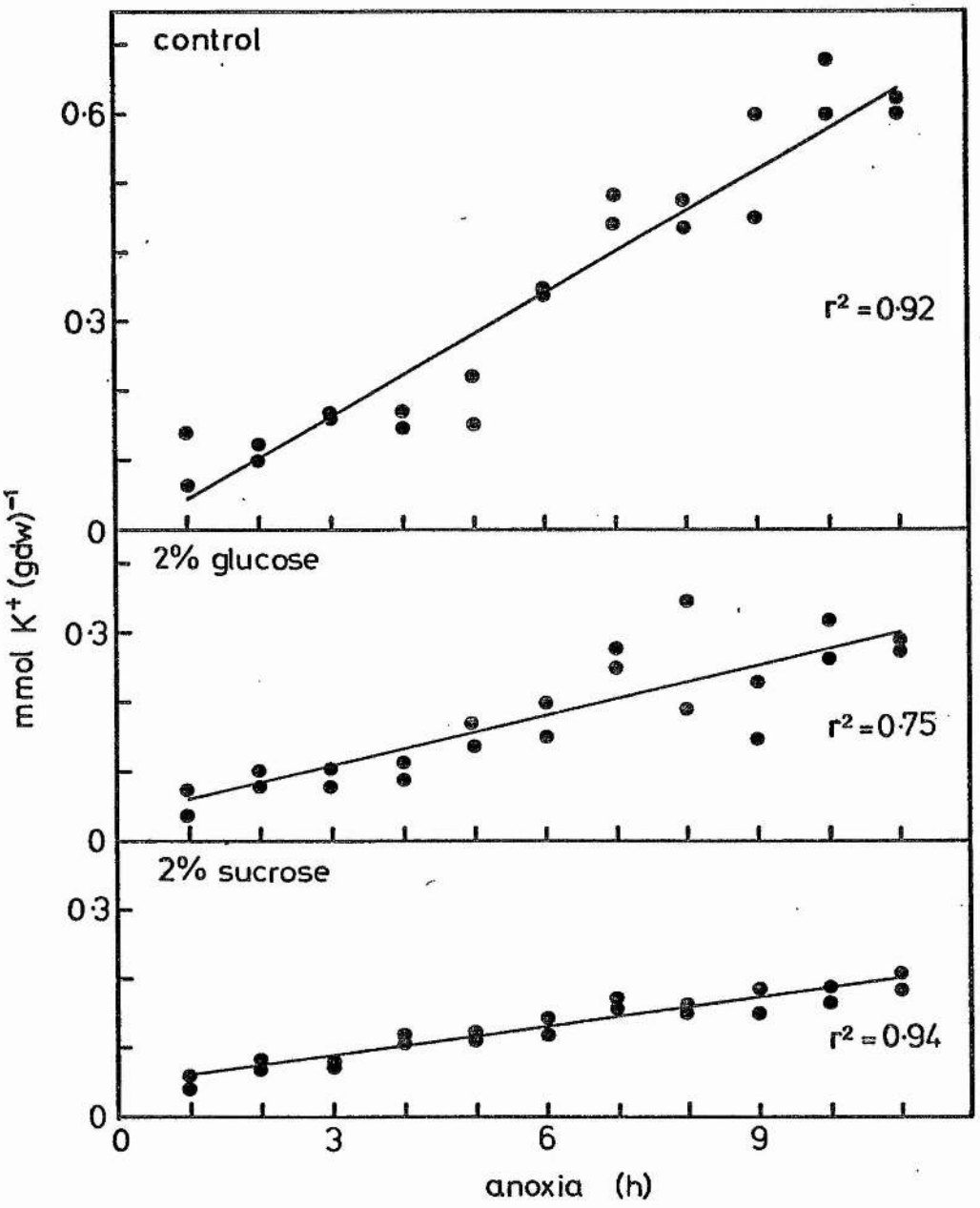


Figure 6.1 The effect of different lengths of time under anoxia on K^+ in the solution around the roots of intact pea seedlings in water, glucose and sucrose solutions.

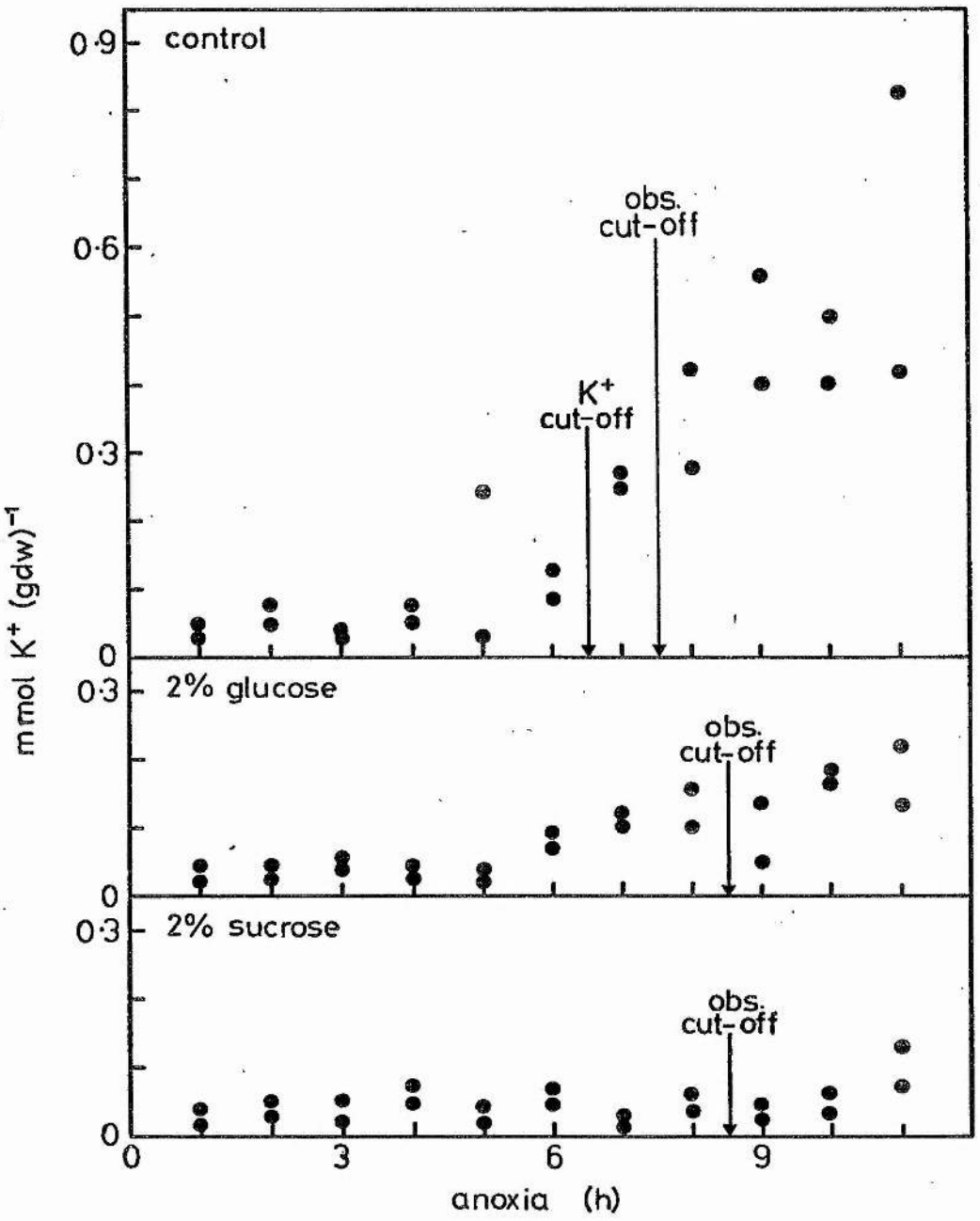


Figure 6.2 The effect of different lengths of time under anoxia followed by a 24 h recovery period in air, on the K^+ remaining in the solution around the roots of intact pea seedlings in water, glucose and sucrose solutions.

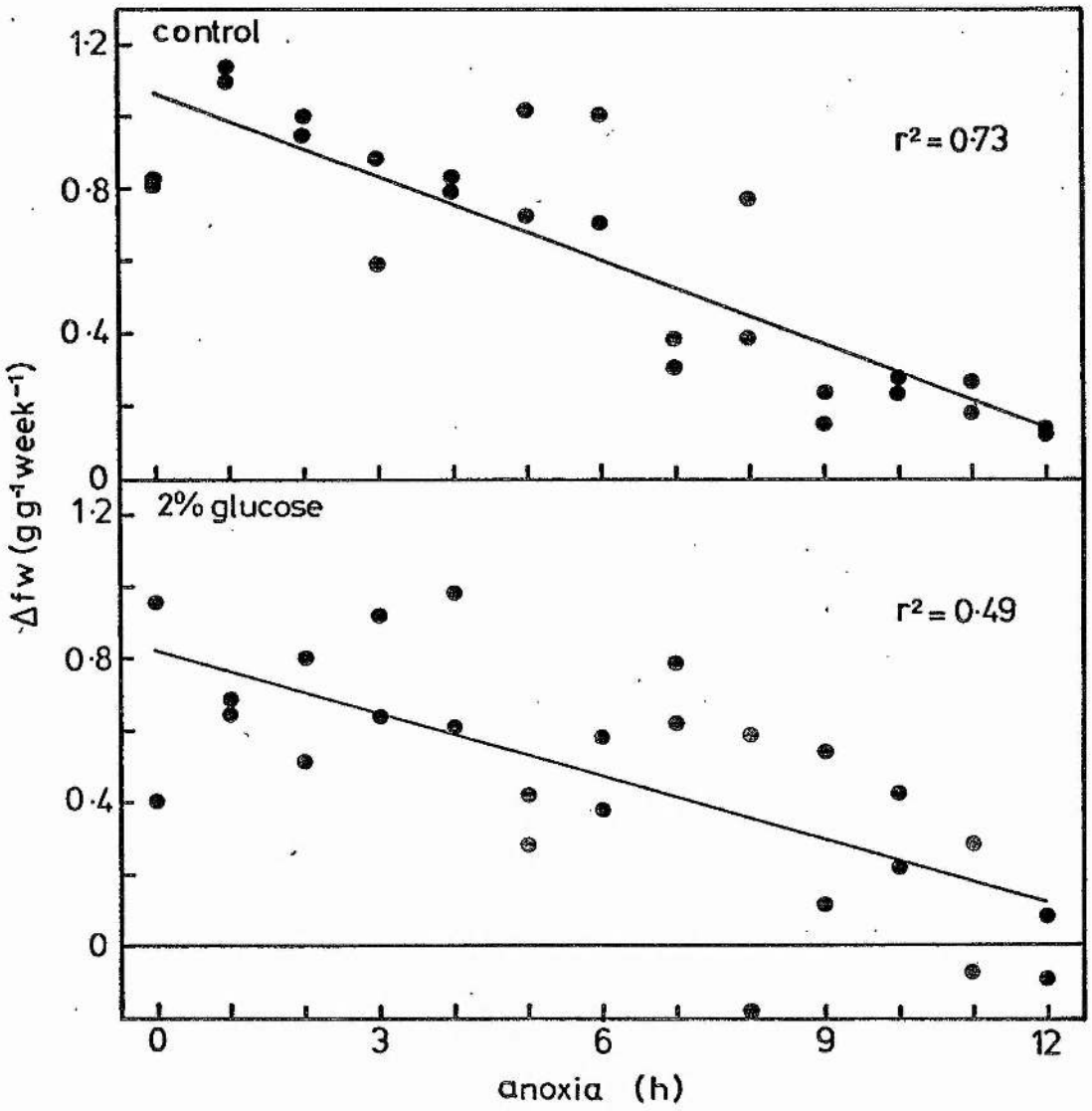


Figure 6.3 The effect of different lengths of time under anoxia in water and 2% glucose solutions on the subsequent changes in fresh weight of pea seedlings after one week in sand.

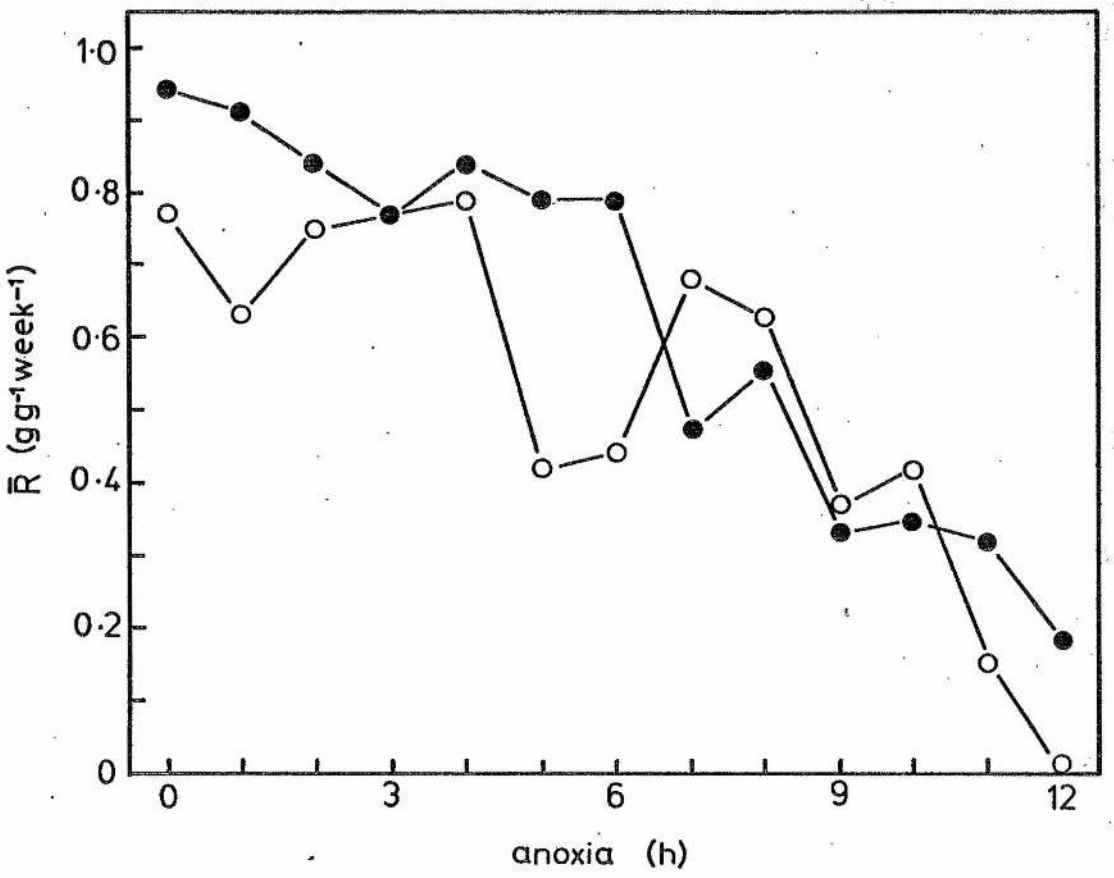


Figure 6.4 Data presented in Figure 6.3, expressed as the mean relative growth rate, $\bar{R}$. (n = 2).

● water; O 2% glucose solutions.

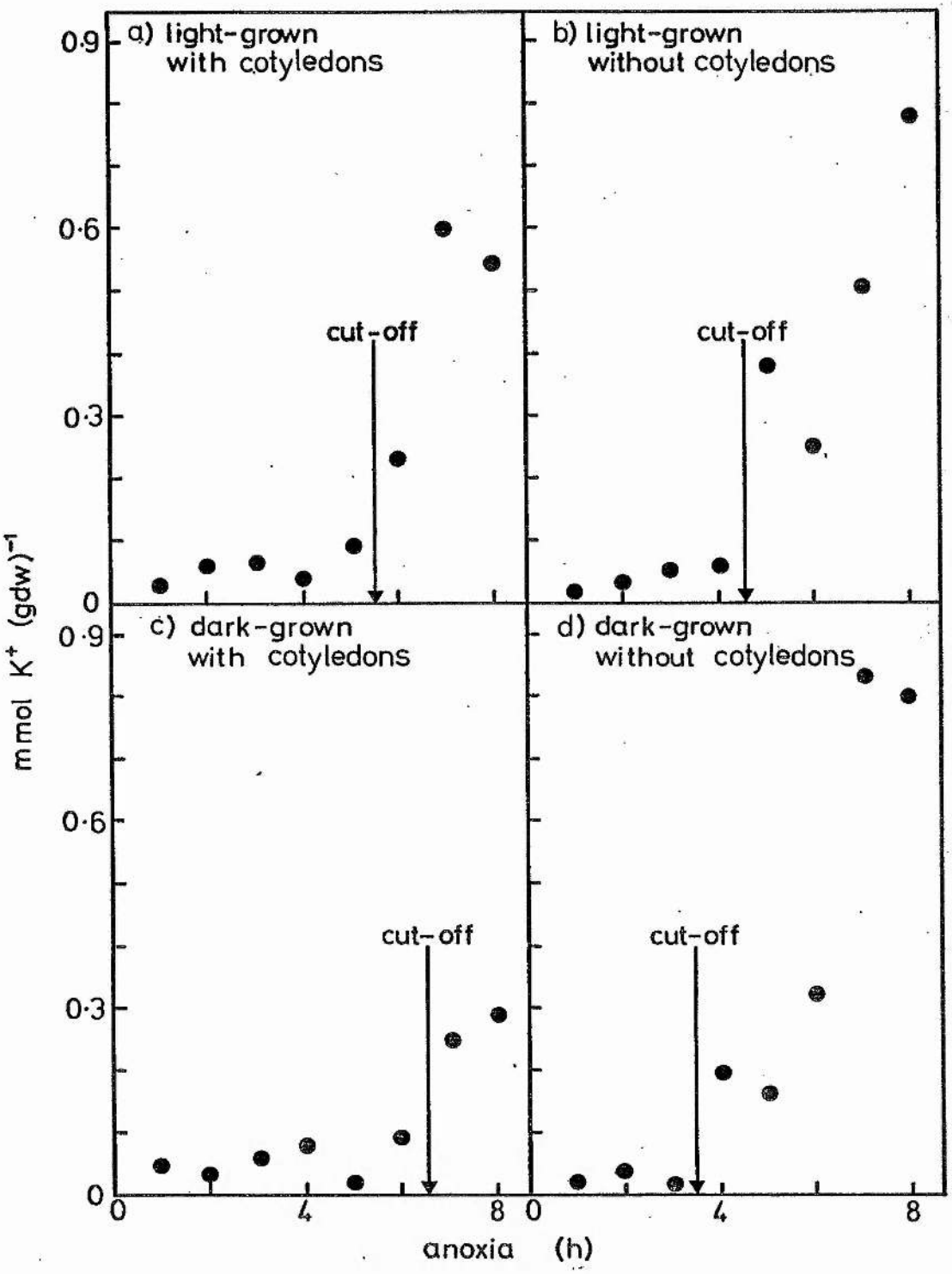


Figure 6.5 The effect of cotyledon removal (b and d) on the time of cut-off by light-grown and dark-grown pea seedlings compared with intact controls (a and c).

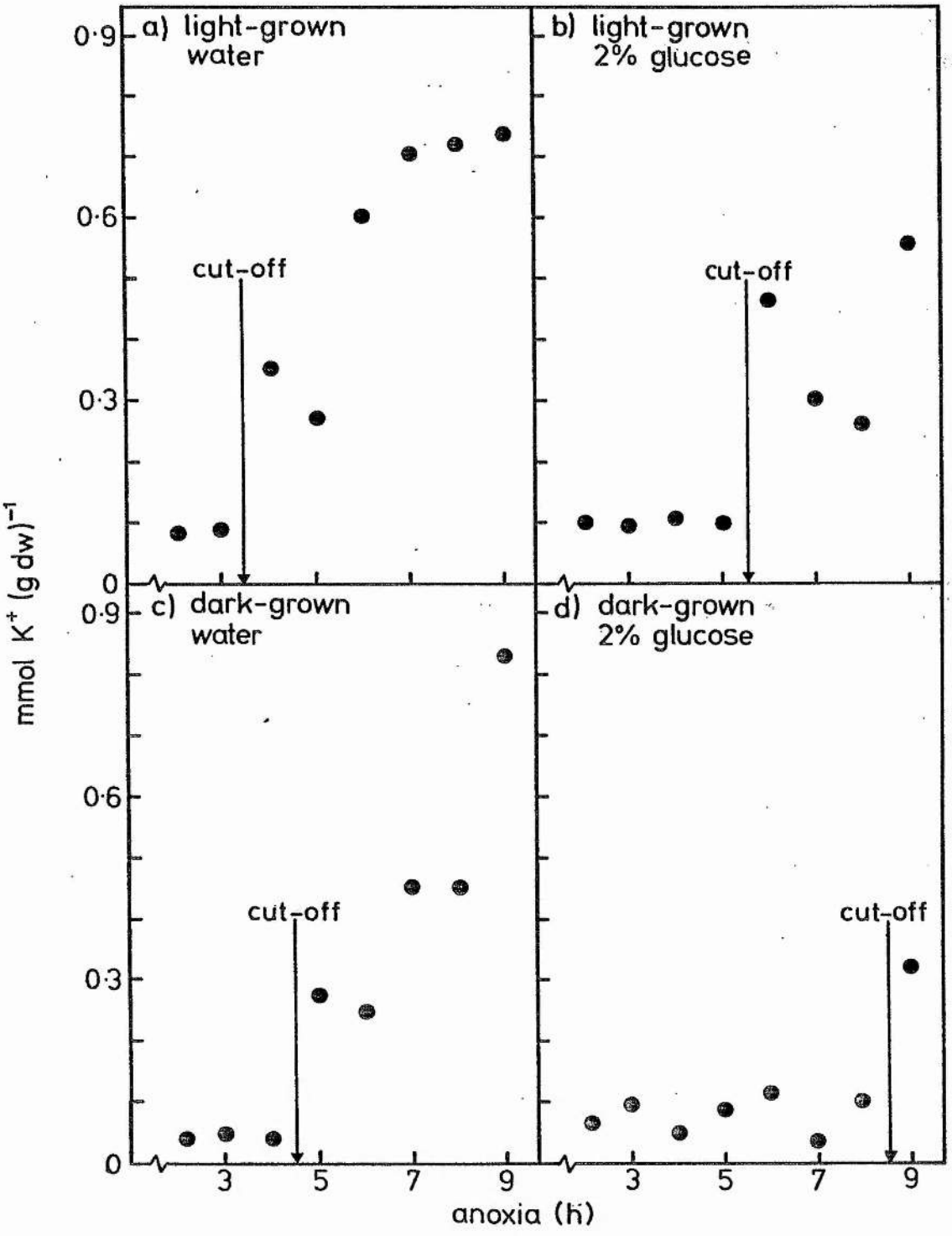


Figure 6.6 The effect of 2% glucose (b and d) on the time of cut-off by light-grown and dark-grown pea seedlings without cotyledons compared with water controls (a and c).

Table 6.1

The effect of cotyledon removal, with and without exogenous glucose, on ethanol accumulation around the roots of pea seedlings after different lengths of time under anoxia (20°C).

Treatment	µmol Ethanol $(gdw)^{-1}$					
	Anoxia (h)					
a) Light-grown	2	4	6	8	10	12
Whole seedling	0.11	0.22	0.38	0.56	0.55	0.61
Cotyledons removed	0.11	0.25	0.38	0.55	0.52	0.52
Cotyledons removed, + 2% glucose	0.10	0.22	0.42	0.45	0.55	0.67
b) Dark-grown						
Whole seedling	0.11	0.21	0.38	0.59	0.63	0.67
Cotyledons removed	0.10	0.34	0.39	0.62	0.45	0.59
Cotyledons removed, + 2% glucose	0.04	0.23	0.42	0.57	0.59	0.46

CHAPTER 7

RICE: VARIETAL DIFFERENCES

7.1 INTRODUCTION

Rice is an important agronomic species which provides food for half of the Earth's population (from Taylor,1942 and Vartapetian et al., 1978), and it is usually grown in paddy fields where 5-10 cm of standing water is maintained during the 4-5 months the crop is in the field (Ponnamperuma,1972). However, there are many different varieties of rice and these are grown under different water regimes in different areas of the world. In many rice-growing areas rain is the main source of water, but because this supply cannot be controlled droughts or floods may occur (IRRI Report,1974), and although rice is regarded as a highly successful flood-tolerant species, the complete submergence of many varieties can result in considerable crop damage and low grain yields (Palada and Vergara,1972).

Floating rice varieties are able to elongate as the water level rises, and at least five million hectares of this type of rice are grown in areas that are annually flooded to depths of more than 2 m for 3-4 months of the year (IRRI Report,1974). However, in monsoon areas, where the water level may rise between 60 and 90 cm per day at the beginning of the season, floating rice crops may be completely submerged if elongation cannot keep up with the rapidly rising water level. In lowland rice fields, where non-floating rice varieties are grown, complete submergence is a rare event, but nevertheless can occur during periods of unusually heavy rains. There has been much research into the development of improved rice varieties (IRRI Reports,1974 to 1978 inclusive) and submergence tolerance is a desirable characteristic in both floating

and lowland rice varieties, since crop losses would be greatly reduced if the plants could survive the period of submergence.

Vartapetian et al. (1978) regard the young rice seedling as a higher plant with a true adaptation to anoxia, although they attribute the survival of adult plants in flooded soils to the ability of the plants to aerate their roots, rather than to metabolic adaptations to anoxia in the root cells. Therefore adult rice plants should be susceptible to complete submergence whereas young rice seedlings should be tolerant of it. However, Palada and Vergara (1972) reported that rice was most susceptible to submergence during the seedling stage, although when seedlings of fifteen rice varieties were submerged for 10 days, the survival of the different varieties ranged between 30 and 100% (IRRI Report, 1977). Submergence may occur more than once during the season, and Datta and Banerji (1974) found that many rice varieties exhibited better resistance to flooding after the first flood. In a recent study of the tolerances of different rice varieties to complete submergence at the seedling stage, Karin (1980) found that rice varieties FR13A (submergence-tolerant) and IR8 (submergence-intolerant) both exhibited 100% survival after 4 days complete submergence, but after 6 days, survival was 98 and 90% for FR13A and IR8 respectively.

When seedlings are submerged they are exposed to an environment in which molecular oxygen is in short supply, and therefore in this respect submerged and anaerobic environments are similar. This chapter presents data on the tolerance limits and survival of 14 day old rice seedlings of varieties FR13A and IR8 under conditions of total anoxia. Measurements of oxygen uptake rates by excised root tips, ethanol accumulation around the roots and the mean relative growth rate, $\bar{R}$, of seedlings after anoxia were also investigated.

7.2 RESULTS

7.2.2 Tolerance of anoxia

Seedlings of rice, cvs. FR13A and IR8, were grown and subjected to anoxia as described in Sections 2.2.2 and 2.3 respectively. After the anaerobic treatments the seedlings were planted out in sand for one week and their mean relative growth rates, $\bar{R}$, calculated following the method in Section 2.2.3. Seedlings of the submergence-tolerant rice, cv. FR13A, appeared healthy and possessed green, turgid shoot systems after all the anaerobic exposures, whereas seedlings of the submergence-intolerant rice, cv. IR8, were of healthy appearance only after anaerobic exposures of 12 h or less - after 14 h anoxia or longer, IR8 seedlings appeared to have been damaged since their shoot systems were yellow and dried. Figure 7.1 shows the effect of different lengths of time under anoxia on the subsequent $\bar{R}$s of the two varieties. FR13A seedlings maintained a constant $\bar{R}$ during the one week in sand following all the anaerobic exposures, but IR8 seedlings exhibited a decline in $\bar{R}$ after only 8 h anoxia. Thus although the IR8 seedlings experiencing between 8 and 12 h anoxia inclusive appeared to have been unaffected by their anaerobic treatments, these exposures had seriously impaired the subsequent growth of the seedlings. It is interesting to note that the effect of anoxia on the $\bar{R}$ of IR8 seedlings was similar to its effect on the $\bar{R}$ of flood-intolerant pea seedlings (Figure 3.5).

7.2.2 Aerobic respiration

The rate of oxygen uptake by excised, sterilised rice root tips was measured as described in Section 5.2.1, except that in this experiment a recovery period of 48 h was used. Figure 7.2 shows the rates of oxygen uptake by FR13A root tips immediately after the anaerobic exposures (a), and after the whole seedling had experienced a recovery period in air (b). The rate of oxygen uptake immediately after removal

from the anaerobic environment ranged between 46 and 105% of the control rate, following anaerobic exposures of up to and including 22 h anoxia. After the recovery period in air all the seedlings recovered and exhibited oxygen uptake rates that were at least 57% of the control rate, suggesting that damage to the aerobic respiration system had not occurred during 22 h anoxia.

Figure 7.3 shows the rates of oxygen uptake by IR8 root tips immediately after the anaerobic exposures (a), and after the whole seedling had experienced a recovery period in air (b). There was a gradual decrease in the rate of oxygen uptake between 0 and 10 h anoxia but the reason for the sudden increase in rates between 12 and 22 h anoxia is not clear. However, after the recovery period in air, seedlings that had experienced 12 h anoxia or more did not exhibit any oxygen uptake, suggesting that irreversible damage to the aerobic respiration system had occurred after 12 h anoxia. This result is similar to those previously obtained for pea and rice, cv. Oeiras, seedlings (Figures 4.3 and 4.4 respectively).

7.2.3 Ethanol accumulation

The ethanol content of the solution around the roots was determined enzymatically immediately after whole seedlings had experienced between 2 to 22 h anoxia inclusive (Sections 2.6.1 and 2.6.2). Figure 7.4 shows that ethanol accumulated in the solutions around the roots of both rice varieties during anoxia, and that between 6 and 16 h anoxia more ethanol accumulated around the roots of the submergence-tolerant rice, cv. FR13A, than around the roots of the submergence-intolerant rice, cv. IR8. This does not necessarily mean that FR13A seedlings produced more ethanol than IR8 seedlings, since varietal differences in the rate of ethanol loss (diffusion) from the roots may affect its rate of accumulation both internally and externally.

7.3 DISCUSSION

Palada and Vergara (1972) reported that more than 80% of Peta rice seedlings survived 6 days of complete submergence, although a photograph of these seedlings showed that they were approximately half the size of the control seedlings. Similarly, although rice varieties FR13A and IR8 had both been shown to exhibit 100% survival after 4 days of complete submergence, tolerant varieties (including FR13A) were generally taller than susceptible varieties (including IR8), both after subjection to complete submergence and after recovery (Karin, 1980). Thus percent survival does not give any indication of the performance of the seedlings after the period of submergence.

In these present experiments, young FR13A seedlings were found to be more tolerant of anoxia than IR8 seedlings of similar age, with respect to their recovery following the anaerobic treatments. Seedlings of both varieties possessed green and turgid leaves after 12 h anoxia, i.e. they all survived these anaerobic treatments, but their $\bar{R}s$ were remarkably different (Figure 7.1). FR13A seedlings maintained a relatively constant $\bar{R}$ during the one week in sand following anaerobic exposures of up to and including 22 h, whereas IR8 seedlings showed a decline in $\bar{R}$ during the same period following anaerobic exposures of more than 6 h, and after 12 h anoxia very little new growth occurred.

If the $\bar{R}s$ in Figure 7.1 are compared with the oxygen uptake results for the two varieties after a recovery period in air (Figures 7.2b and 7.3b for FR13A and IR8 seedlings respectively), it can be seen that the continued aerobic respiration by FR13A seedlings after all the anaerobic exposures is accompanied by a relatively constant $\bar{R}$ during one week in sand, whereas the cessation of aerobic respiration by IR8 seedlings after 12 h anoxia is accompanied by very little growth during one week in sand after this time. These results suggest that aerobic

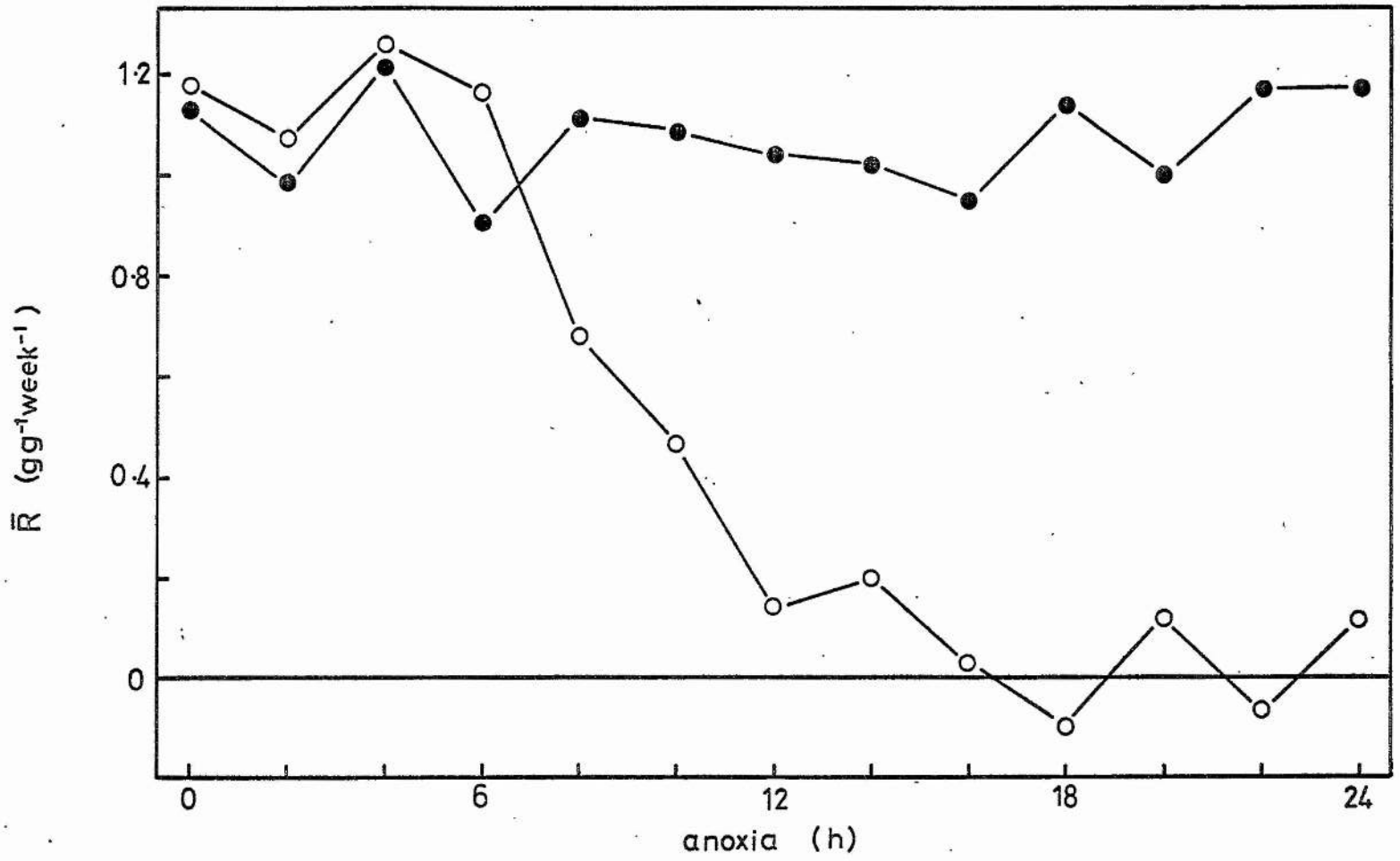
respiration is necessary for seedling recovery after anoxia and are therefore similar to the results obtained for pea and rice, cv. Oeiras, seedlings (Section 4.2.3). Under conditions of root anoxia only, as would occur when the roots are in flooded soils, the tolerance and survival of both FR13A and IR8 seedlings would be expected to be similar because under these conditions rice plants have the capacity to transport oxygen from the shoots to the roots (Barber et al.,1962; John et al.,1974; Vartapetian et al.,1978). This allows aerobic conditions to be maintained in most, although not necessarily all, regions of the roots. In addition,internal ventilation would aid the oxidation of any ethanol not removed from the roots by exudation or transpiration.

Vartapetian et al.(1978) found that when intact rice plants were subjected to anoxia, root ultrastructure remained unchanged for 3 days. However, when detached roots were subjected to anoxia, mitochondria began to degrade after 1 day, but detached roots that were subjected to anoxia in 0.5% glucose solutions maintained an undamaged ultrastructure for 3 days. Thus they concluded that sufficient stores of organic substances in the cell are essential for the maintenance of cell ultrastructure during anoxia. Indeed growth of the rice coleoptile during anoxia is thought to be sustained by a supply of organic compounds translocated from the grain to the coleoptile during the anaerobic period (Vartapetian et al.,1978).

In these present experiments there was a marked difference between the tolerances of FR13A and IR8 seedlings to anoxia. Palada and Vergara (1972) found that the amount of starch in Peta rice seedlings decreased with submergence, presumably because photosynthesis was reduced, and that plants with more initial carbohydrate reserves were better able to survive periods of submergence than plants with lower initial reserves. Karin (1980) found that submergence-tolerant rice varieties (including

FR13A) had heavier grains than submergence-intolerant varieties (including IR8), suggesting that submergence-tolerant varieties possessed more organic reserves than submergence-intolerant varieties. Therefore it is possible that FR13A seedlings were more tolerant of anoxia than IR8 seedlings because they possessed larger quantities of glycolytic substrate, and thus were able to maintain an adequate supply of ATP during the period of anoxia investigated. However, the decline in $\bar{R}$ of IR8 seedlings between 6 and 12 h anoxia may have been caused by the accumulation of ethanol within the tissues, although irreversible damage to the aerobic respiration system could have been caused either by the accumulation of a toxic quantity of ethanol, or by a shortage of glycolytic substrate, and hence ATP (see Section 6.3). The roots of FR13A seedlings may have been more permeable to the diffusion of endogenous ethanol than the roots of IR8 seedlings, or alternatively FR13A seedlings may have had a higher tolerance of endogenous ethanol than IR8 seedlings (see Section 5.3).

Figure 7.1 The effect of different lengths of time under anoxia on the subsequent mean relative growth rates, $\bar{R}$, of rice seedlings after one week in sand. (n = 2). ● cv. FR13A, O cv. IR8; 25°C.


1·2
0·8
0·4
0
$\bar{R}$ (gg^{-1}week^{-1})
0
6
12
18
24
anoxia (h)

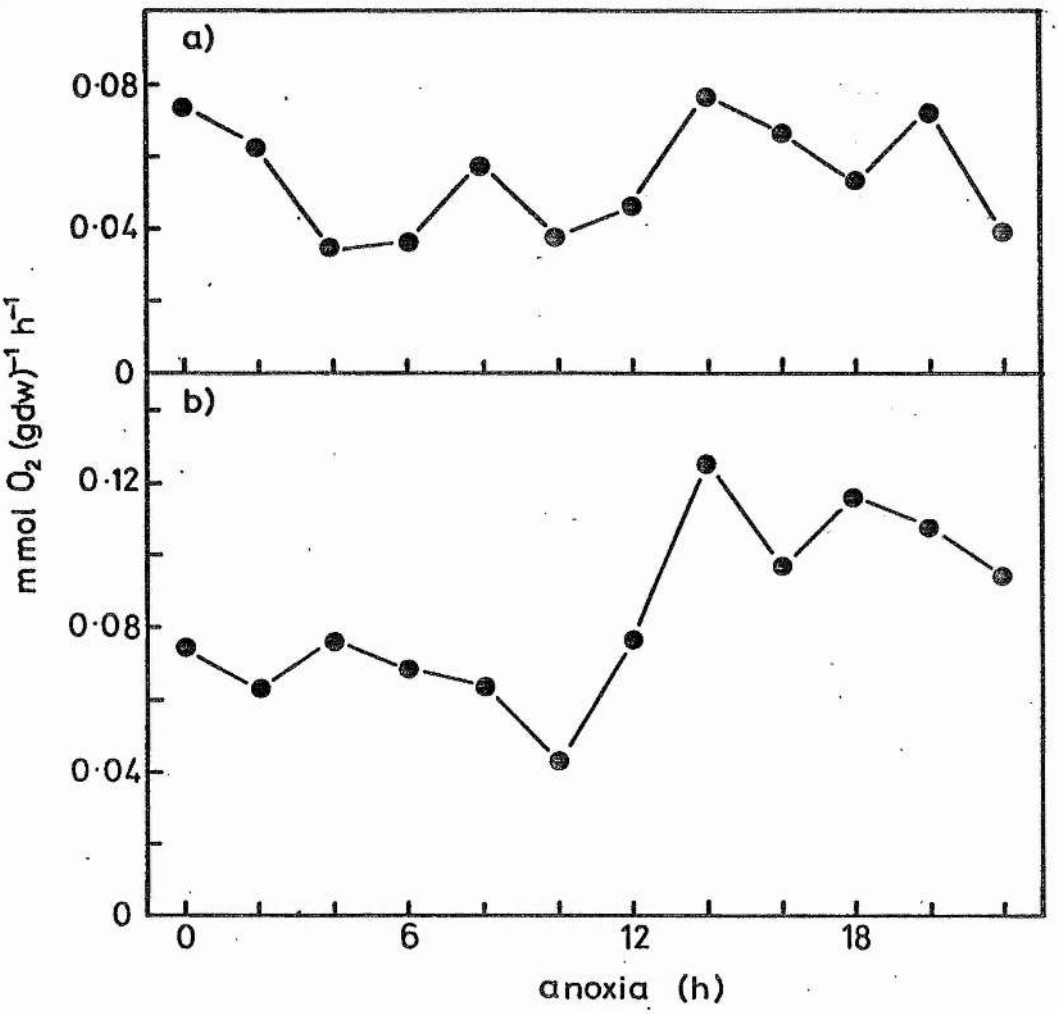


Figure 7.2 The effect of different lengths of time under anoxia on the oxygen uptake rates of excised, sterilised rice root tips, cv. FR13A (25°C).

(a) Immediately after anoxia.

(b) After a 48 h recovery period in air.

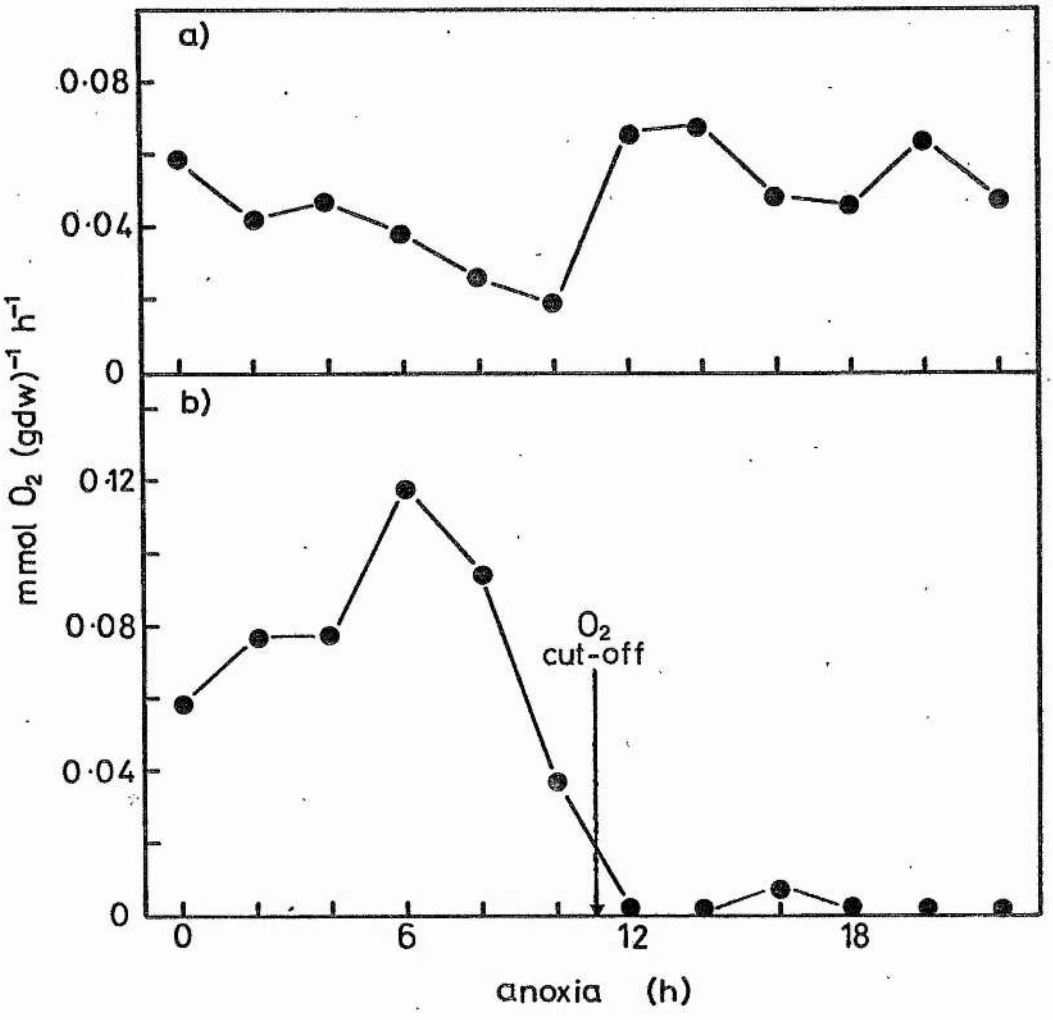


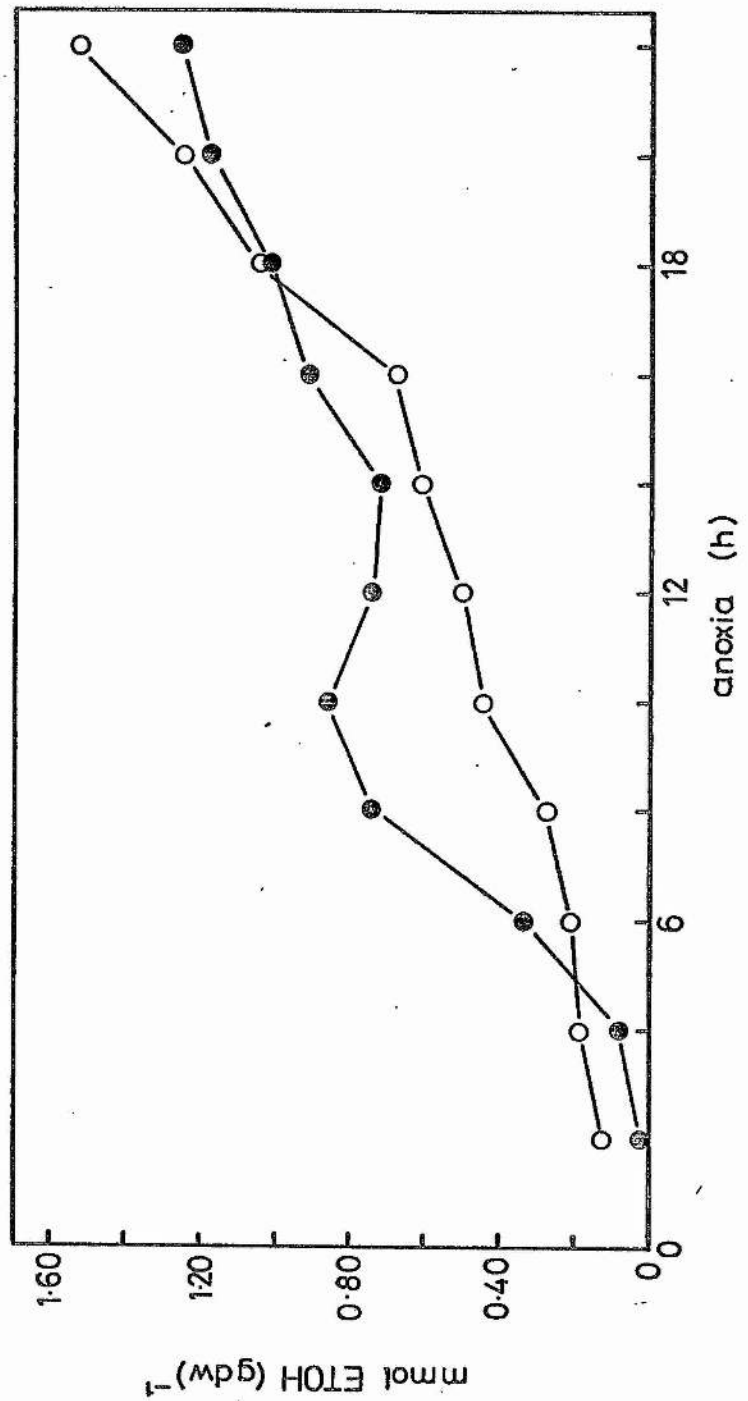
Figure 7.3 The effect of different lengths of time under anoxia on the oxygen uptake rates of excised, sterilised rice root tips, cv. IR8 (25°C).

(a) Immediately after anoxia.

(b) After a 48 h recovery period in air.

Figure 7.4 The effect of different lengths of time under anoxia on ethanol accumulation in the solution around the roots of intact rice seedlings at 25°C.

● cv. FR13A, ○ cv. IR8.


mmol ETOH (gdw)$^{-1}$
1·60
1·20
0·80
0·40
0·0
0
6
12
18
anoxia (h)

CHAPTER 8

DISCUSSION

Tolerance of anoxia

Flooding the soil may adversely affect the growth of a number of plants, including many crop species, which normally grow in well-drained soils. Although the responses of plants to soil flooding are well documented (see reviews by Bergman,1959; Grable,1966; Drew and Lynch,1980), the actual causes of injury in flood-intolerant species are still unclear. Experiments in anaerobic solution cultures, where the effects of potential soil toxins have been excluded, have shown that the exclusion of oxygen from the root environment of many flood-intolerant species was sufficient to cause symptoms of flooding injury (Letey et al., 1962; Willey,1970; Drew and Sisworo,1979; Trought and Drew,1980a and b), possibly as a result of the impairment of aerobic respiration in the roots (Lemon and Wiegand,1962). The experiments reported in this thesis compare the responses of pea and rice seedlings to a completely anaerobic environment in deoxygenated solutions, where internal ventilation is prevented and soil toxins are excluded.

Experiments in Chapters 4 and 7 showed that in seedlings of pea and three varieties of rice, the resumption of aerobic respiration after anoxia was essential for seedling survival. These results, originally presented graphically in Sections 4.3 and 7.3, are in Table 8.1 expressed as the mean values before and after the cut-off, and they show that reduced rates of oxygen uptake after the recovery period were associated with reduced mean relative growth rates, $\bar{R}$, following the anaerobic treatment. Each species or variety had a different tolerance limit under anoxia, and when this limit was exceeded irreversible damage to

the aerobic respiration system occurred, ultimately resulting in seedling death. Similar results were found for Triticale sp. (Oliveira,1977), Cucurbita pepo (Coulomb and Coulomb,1972b, cited by Oliveira,1977) and Lycopersicum esculentum (Morisset,1978)

Ethanol toxicity

Oliveira (1977) suggested that the loss of aerobic activity may be connected with the degradation of mitochondria during anoxia. The enzymes of aerobic respiration, i.e. those of the TCA cycle and oxidative phosphorylation, are contained in membrane-bound mitochondria, whereas the enzymes of glycolysis and those involved in the conversion of pyruvate to ethanol are located in the cytoplasm. Ethanol is thought to have its toxic effect by attacking and fluidising cell membranes (Kiyosawa,1975; Nandini-Kishore et al.,1979) and so its production and / or accumulation in the cytoplasm near mitochondria during anoxia may damage these organelles (Crawford,1977). Indeed Lenaz et al. (1971) found that phosphorylation in beef heart mitochondria was 50% inhibited by 0.77 mol l^{-1} ethanol. Alternatively, the degradation of mitochondria may occur during anoxia because of the inhibition of oxidative phosphorylation, which normally provides the ATP essential for the maintenance of mitochondrial integrity (Luzikov et al.,1973).

All the seedlings accumulated ethanol in the solutions around the roots during anoxia, but there was no correlation between the quantity of ethanol produced per gramme dry weight of root tissue and the different tolerance limits of the seedlings to anoxia. Unfortunately it is not known to what extent ethanol actually accumulated within the roots of the different seedlings during anoxia. Ethanol is an uncharged molecule and Davies (1958, cited by Davies,1980) argued that as a result it should be readily lost from cells, as are other uncharged, end-products of metabolism, e.g. urea. However, although there are many reports in

the literature that ethanol is lost from plant tissues to the surrounding medium during anoxia (Grineva,1962; Aubertin et al.,1966; Bolton and Erickson,1970; Bertani et al.,1980; Rumpho and Kennedy,1981), there are also numerous references concerning the accumulation of ethanol within plant tissues, including roots, during anoxia (Fulton and Erickson,1964; Grineva,1964; Leblova et al.,1969; Andrews,1977; Crawford and Baines, 1977; Chirkova,1978; Barclay and Crawford,1981).

If ethanol readily diffuses out of root cells, then it is reasonable to assume that there will be a net diffusion into root cells if the roots are subjected to external ethanol concentrations higher than the ethanol concentrations within the tissue. Thomas et al. (1978) suggested that the death of Saccharomyces cerevisiae cells under aerobic conditions in 1.0 mol l^{-1} (1M) ethanol may have resulted from the denaturation of intracellular enzymes following the diffusion of ethanol into the cells. Results in Sections 5.2.4 and 5.2.5 showed that concentrations of ethanol up to 0.1 mol l^{-1} were not toxic to pea and rice, cv. Oeiras, seedlings since neither the subsequent growth of the seedlings in sand (Figures 5.6 and 5.7), nor the tolerance limit of pea seedlings to anoxia (Figure 5.8), were adversely affected by this concentration. An ethanol concentration of 0.3 mol l^{-1} caused the pea seedling cut-off to occur 2 h earlier than the controls(Figure 5.8c and d), and in another experiment it significantly reduced the subsequent growth of seedlings after a 24 h exposure of the roots to this concentration in air (Table 5.1). However, this concentration of ethanol is much higher than the concentrations reported to accumulate in either flooded soils (Wang and Chuang,1967; Jackson,1979), or in the solutions around the roots of pea and rice seedlings subjected to anoxia (Section 5.3). Nagodawithana and Steinkrauss (1976) found that in a fermenting culture of Saccharomyces cerevisiae the internally produced ethanol was considerably more toxic than

that applied externally, and therefore it is possible that a similar situation exists in higher plants in an anaerobic environment.

Glycolytic substrate supply

In many plant tissues the consumption of glycolytic substrate increases during anoxia (see Turner,1951) but since the anaerobic breakdown of 1 mole of glucose produces only 2 moles of ATP compared with the 36 moles of ATP from the aerobic breakdown (Beevers,1961), there is a reduction in the rate of ATP production during anoxia. Thus seedling death under anoxia may occur if the supply of ATP is insufficient for the maintenance of cellular processes during the anaerobic period. The ATP supply during anoxia may be insufficient either because the glycolytic substrate becomes exhausted, or because the rate of ATP production cannot satisfy the demand.

Vartapetian et al. (1977) showed that exogenous glucose was beneficial to excised rice and pumpkin roots during anoxia, and they concluded that carbon starvation may be the cause of damage to root cell ultrastructure during anoxia. In these present experiments, pea and rice seedling recovery after anoxia was associated with the capacity for aerobic respiration. Therefore in these seedlings damage to the mitochondria, the sites of aerobic respiration, during the anaerobic period either did not occur or was reversible. ATP is essential for the maintenance of mitochondrial integrity (Luzikov et al.,1973) and thus it follows that up to the time of the cut-off there was sufficient ATP available for this during anoxia. The cut-off by pea seedlings was thought to have been caused by a shortage of glycolytic substrate (see Section 6.3), which through its effect on the ATP supply, ultimately resulted in the degradation of mitochondria, hence the loss of oxygen uptake ability on return to aerobic conditions. A shortage of substrate for glycolysis may have occurred as a result of an increase in glycolytic

rate, which coupled with the inhibition of translocation during anoxia, would rapidly deplete cell reserves (see Vartapetian et al.,1978).

Two percent glucose may have delayed the onset of damage in pea seedlings because it provided substrate for glycolysis and thereby prolonged both the production of ATP and the integrity of mitochondria. However, 2% glucose merely delayed and did not prevent the onset of damage, although the quantity of exogenous glucose was probably not limiting (see Section 6.3). Under these conditions the cut-off, which occurred after 9 h anoxia in this experiment, could have been caused either because a toxic quantity of ethanol had accumulated, or because the maximum rate of ATP production during anoxia was insufficient to maintain mitochondrial integrity for more than 9 h. Since in each case the result would be irreversible damage to mitochondria, with the impairment of aerobic respiration and growth on return to air, the actual cause of seedling death in the presence of 2% glucose is extremely difficult to determine.

In germinating seeds, where glycolytic substrate may be considered non-limiting because of the carbohydrate reserves in the seed, the accumulation of toxic quantities of ethanol rather than a shortage of glycolytic substrate may be the cause of seed deterioration or seedling death under anoxia. Barclay and Crawford (1981) found that the survival of 3 day old pea seedlings at different temperatures under anoxia was always associated with an internal ethanol concentration of less than 60 μmol l^{-1}, and Woodstock and Taylorson (1981) found that deterioration of soybean seeds during accelerated aging was accompanied by increased levels of acetaldehyde and ethanol in imbibing seeds. Therefore, ethanol accumulation may be the more likely cause of the cut-off by whole pea seedlings in 2% glucose solutions.

Delayed growth responses to anaerobic damage

It is interesting to note that although some of the pea and rice, cv. IR8, seedlings recovered from their anaerobic treatments, i.e. they did not show immediate signs of damage after removal from the anaerobic environment, their subsequent growth in sand had been seriously impaired (Figures 3.5 and 7.1 for pea and rice cv. IR8 respectively). In contrast to this, seedlings of rice cvs. Oeiras and FR13A maintained a relatively steady $\bar{R}$ after 14 h and at least 22 h anoxia respectively (Figures 3.5 and 7.1 for cvs. Oeiras and FR13A respectively). Unfortunately the effect of exogenous glucose on the survival of whole rice seedlings during anoxia was not investigated, but nevertheless the cut-off by rice cvs. Oeiras and IR8 may also have been caused by a shortage of glycolytic substrate. It is suggested that rice, cv. FR13A, seedlings had sufficient carbohydrate reserves to maintain a rate of glycolysis that satisfied the ATP requirment of the seedlings for at least 22 h anoxia, whereas rice, cvs. Oeiras and IR8, seedlings may have exhausted their organic reserves after 12 and 10 h anoxia respectively, hence the loss of aerobic activity in the excised roots (Figures 4.4b and 7.3b for cvs. Oeiras and IR8 respectively). However, although the tolerance limits of these two rice varieties under anoxia were similar, their subsequent $\bar{R}$s after the cut-off were remarkably different (Figures 4.5 and 7.1 for cvs. Oeiras and IR8 respectively).

Vartapetian et al. (1978) found that rice root cells were less tolerant of anoxia than coleoptile cells, which unlike the root cells received glycolytic substrate translocated from the grain, and so were able to maintain an undamaged ultrastructure during longer periods of anoxia. Therefore in these present experiments the oxygen uptake ability of the root tips may not have reflected the condition of the coleoptile cells. Thus it is possible that the continued growth of rice cv. Oeiras

seedlings after 14 h anoxia was solely a result of shoot growth (see Section 3.3). However, in rice cv. IR8 seedlings shoot growth may have been prevented because glycolytic substrate in the grain had been exhausted It is interesting to note that the seeds of rice cv. FR13A, the submergence-tolerant variety, were heavier than the seeds of rice cv. IR8, the submergence-intolerant variety (Karin,1980), suggesting that FR13A seeds contained more reserves than IR8 seeds.

Ethanol removal

Between 0 and 12 h anoxia the $\bar{R}$ of rice cv. IR8 seedlings was similar to that of pea seedlings, since in both cases the growth rate declined up to the time of the cut-off, although the seedlings appeared undamaged and maintained the capacity for aerobic respiration in the root tips. Bertani et al. (1980) attributed the survival of rice seedlings during anoxia not only to their ability to develop a vigorous fermentation system, but also to their ability to remove most of the ethanol (98% in their experiments) from the tissues. It is possible that in pea and rice, cv. IR8, seedlings the accumulation of ethanol within the tissues during anoxia caused increasing damage to cellular activities, e.g. enzyme systems (Thomas et al.,1978), so that on return to aerobic conditions the original rates of cellular activities and growth could not be attained for some time. Such a situation may not have arisen in rice seedlings, cvs. Oeiras and FR13A, because ethanol was efficiently removed from the tissues by diffusion and transpiration during anoxia, hence the steady $\bar{R}$s of Oeiras and FR13A seedlings after 14 and 22 h anoxia respectively. Since Ingram (1976) found that Escherichia coli K-12 cells changed their fatty acid composition in the presence of alcohol, and Thomas et al. (1978) found that the tolerance of Saccharomyces cerevisiae to exogenous ethanol was related to the lipid composition of the plasma-membrane, it is possible that the plasma-membrane composition of pea and rice root cells

differed with respect to the permeability of ethanol.

Ethanol accumulated in the solutions around the roots of pea and rice seedlings during anoxia, but the quantities of ethanol exuded by the different species or varieties are meaningless unless it is known what proportion of the total ethanol production is represented in each case. Evidence for the accumulation of a toxic substance within pea seedlings during anoxia comes from the transpiration experiments (Section 5.2.3). Seedlings that were prevented from transpiring during the anaerobic treatment exhibited symptoms of damage 2 h earlier than the transpiring control seedlings, presumably because the accumulation of the toxin had been accelerated. The cut-off by pea seedlings in 2% glucose solutions may have been caused because ethanol reached a concentration high enough to damage mitochondria, although the possibility that the rate of ATP supply during anoxia was insufficient to maintain mitochondrial integrity cannot be dismissed.

Conclusions

Under natural conditions, plants are seldom exposed to a completely anaerobic environment, although the roots of some species may experience a period of oxygen deficiency when flooded. Many plants, including a number of flood-intolerant species, have some capacity for internal ventilation (Armstrong, 1978) and so in order to totally exclude oxygen from the root environment it is necessary to exclude oxygen from around both the roots and the shoots. In some species, flooding the roots may result in the death of the entire root system, but not necessarily the plant, since new adventitious roots often grow to replace the original root systems (Kramer, 1951). Such new growth would depend on the presence of oxygen and on a supply of photosynthate and other substances which had been synthesised in the shoots. However, when the whole plant is subjected to anoxia, the additional effect of oxygen deficiency on the

shoot system may further reduce the probability of seedling recovery from the anaerobic treatment. In these present experiments seedlings were subjected to anoxia in the dark and therefore both aerobic respiration and photosynthesis were prevented during the period of anoxia. However, although there are many differences between the conditions in the present experiments and those in the field, the cause of root death may be the same in both cases.

From the results presented in this thesis and elsewhere, it is concluded that seedling death during anoxia arises from a shortage of glycolytic substrate, which leads to a cessation of ATP production and a loss of cellular integrity. Ethanol may accumulate in the roots of some species and although it may not kill the plant, it may reduce its rate of recovery after the period of anoxia.

Table 8.1

Times of cut-off under anoxia and the subsequent rates of oxygen uptake and mean relative growth rates, $\bar{R}$, of pea and rice seedlings before and after the cut-off. Values are means of data in Chapters 4 and 7.

Species	Temperature (°C)	Time of cut-off under anoxia (h)	$\mu mol\ O_2\ (gdw)^{-1}\ h^{-1}$		$\bar{R}\ (g\ g^{-1}\ week^{-1})$	
			Before cut-off	After cut-off	Before cut-off	After cut-off
Pea	20	6 - 8	0.12	0.03	0.60	0.00
Rice cv. IR8	25	10 - 12	0.08	0.00	0.97	0.06
cv. Oeiras	25	12 - 14	0.09	0.00	1.00	0.60
cv. FR13A	25	after 22	0.08	0.08	1.05	1.05

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